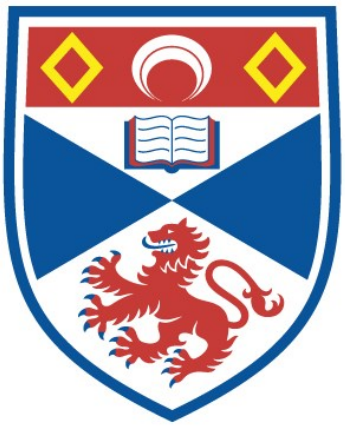
PLASMA PROTEIN CHANGES IN OPEN CARDIAC SURGERY

Allan Henry Lloyd

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1970

Plasma Protein changes in open cardiac surgery.

by

Allan Henry Lloyd

A thesis

presented to the University of St. Andrews for the Degree of

Doctor of Philosophy.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Declaration.

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition, and that no part of it has been presented previously for a higher degree.

The research was carried out in the Department of Biochemistry in the United College of St. Salvator and St. Leonard, St. Andrews, under the direction of Professor G.R. Tristram.

C e r t i f i c a t e.

I hereby certify that Allan M. Lloyd has spent nine terms engaged on research work under my direction, and that he has fulfilled the conditions of Ordinance General No. 12 since October 1966 and from February 1st 1967 under Resolution of the University Court No. 1, and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

[redacted]

Academic Record.

I graduated M.B., Ch.B., in July 1965 at the University of Bristol and matriculated at the University of St. Andrews in October 1966. Since when I have been engaged in research, the results of which are now submitted for the Degree of Doctor of Philosophy.

For one year I held a Medical Research Council Grant as a research student. In July 1967 I was appointed to the Staff as Medical Demonstrator in the Department of Biochemistry in United College.

Acknowledgements.

I would like to express my appreciation for all the help and guidance given to me by Professor G.R. Tristram, Mr. W.F. Walker (Consultant Surgeon - Dundee Royal Infirmary) and to Professor D.M. Douglas for facilities accorded in his Department.

I am indebted to the Medical Research Council for financial support during the first year of research.

I would also like to thank Mr. E. Carstairs for amino acid analyser data, Mr. W. Blyth for the photographs, Mr. J. Crawford for ultracentrifuge studies, Mrs. J. Galloway and Mrs. P. Pearce for the typing, and to many technicians and students for their willing donation of control blood samples.

Foreword.

The aim of this work has been to investigate plasma protein and amino acid changes in blood and urine that take place during open cardiac surgery in operations lasting 30 minutes to over 3 hours. These changes together with allied results are compared with ordinary operations in which extra corporeal circulation is not needed.

In a preliminary procedure blood changes were investigated using the blood oxygenator machine and ancillary equipment only, thus enabling a quantitative comparison to be made between 1) the blood changes due to the oxygenator machine alone and 2) blood changes occurring in normal open cardiac surgery employing an oxygenator utilising the same principle of oxygenation.

Due to the stress the operation imposes upon the patient, hormonal studies were also investigated pari passu with protein and amino acids. The direct hormonal effect on the latter factors is also discussed.

The cause and significance of the amino acid increase in plasma and urine has been investigated.

The change in plasma proteins themselves has been investigated with emphasis on denaturation - as a cause of 1) amino acid increase, 2) increased stress/shock during this type of operation thus being a vital factor in morbidity and mortality statistics.

The results of this work show the quantitative changes that take place in open heart surgery and the many affinities with blood and urine changes in general surgery.

C O N T E N T S.

Abbreviations.

A.I.	Aortic incompetence.
ASD.	Atrial septal defect.
AVR.	Aortic valve replacement.
D.T.N.B.	5'5 Di thio bis (2 nitro benzoic acid).
E.A.C.A.	ϵ - amino caproic acid.
E.P.	End point.
F.P.T.	Full perfusion time.
M.V.R.	Mitral valve replacement.
M.S. and I.	Mitral stenosis and incompetence.
N.E.M.	N- Ethyl maleimide.
P.C.M.B.	P. chloro mercuri benzoic acid.
P.N.P.D.	P. nitrophenyl disulphide.
V.S.D.	Ventricular septal defect.

I N T R O D U C T I O N.

Open heart surgery using cardiopulmonary bypass (extra corporeal circulation) has now become commonplace in many countries. Much research has been done on physiological and to some extent biochemical changes taking place in these operations. The earliest physiological and fluid and electrolyte balance studies have come from the United States, viz:-

Dennis et al., 1951; De Wall et al., 1956 and 7; Stephenson et al., 1956; Baer et al., 1958; and from Scandinavia, viz:-

Craafoord et al., 1956 and 7; Norlander et al., 1958; Husfeldt, 1961; Norberg et al., 1960; and Björk et al., 1961; and in Great Britain the comprehensive, concise study of Walker et al., 1963.

Little work however has been reported on the plasma protein and amino acid changes which occur during perfusions. Due to the duration, morbidity and mortality of these operations it has become essential for these latter changes that take place to be more thoroughly investigated.

Preliminary reports on plasma protein changes in extra corporeal circulation have come from Wright et al., 1962; and Lee et al., 1961. Both these workers only discuss plasma protein changes occurring in the screen and membrane oxygenators and then come to widely different conclusions

about the rate of denaturation. Viscosity and turbidity changes are mentioned but the significance of the findings not discussed. As will be shown in the following work changes in blood due to circulation in the oxygenator and pump circuit alone, are different from blood changes seen in open cardiac perfusion studies, and the changes recorded due to differing unrelated factors.

Similarly, although sulfhydryl analysis of plasma was undertaken in the above work, the significance and relation if any to concomitant haemolysis was not discussed. The viscosity changes are measured to Staudinger units and thus direct comparison in centipoise terms is difficult.

In this type of work full comprehensive elucidation of blood changes can only be obtained by comparing the 'in vitro' changes of the oxygenator-pump circuit with the 'in vivo' changes of open cardiac surgery in which details such as age, sex, height, weight, duration of perfusion, drugs administered etc. are taken into account as well.

Dimililer and Trout, 1965, discussed in a preliminary report the plasma and urinary amino acid changes in cardio pulmonary bypass, but only the increase in free amino acids was mentioned - acid hydrolysis of samples to yield the total increase not being undertaken. No mention is made of the age or sex of the patient, the individual durations of operation, nor in fact the drugs used - especially steroids during the perfusion. It is interesting that an

?unidentified amino acid was found during perfusion - between proline + serine - but since no mention was made of the solvent systems used in the T.L.C. (thin layer chromatography) separation it was impossible to hazard a guess as to what this spot was, - assuming it is an amino acid and not an aliphatic amine or non aromatic heterocyclic compound!

It is necessary and advisable at this stage of the introduction to mention the type of operations with which this study has been concerned.

Operations of varying duration (30 minutes to 3¾ hours) were performed on patients (5 to 65 years) who were suffering from cardiac disease or abnormality necessitating open cardiac surgery. The cardiac aetiology was of congenital and acquired types, the former being atrial/ventricular septal defects and the latter rheumatic heart disease although one case was syphilitic.

Open cardiac surgery requires mechanical oxygenation of the blood which is done outside the patient in the extra corporeal circulation by the Rygg-Kyvsgaard oxygenator bag described below, - (with the other two types of oxygenators which can be used instead). It has become necessary to investigate more fully the changes which take place in the blood (and urine) during full perfusion as a direct result of this form of oxygenation, and compare with operations (general surgery) in which it is not used.

The term full perfusion means that the total blood volume (perfusate) is being continuously and wholly circulated through both patient and extra corporeal circuit (described below).

Partial perfusion signifies that only part of the patients blood volume is passing through the extra corporeal circuit to be oxygenated. This takes place before and after full perfusion when the pulmonary oxygenation is being respectively reduced or re-commenced. This procedure is of short duration - 5-20 minutes.

Amino Acid changes.

It is possible that due to (a) the prolonged operational procedure and (b) the effect of oxygenation of blood, that patients may suffer from renal damage and may die from renal failure due to the liberation of toxic products)? The clinical interest in this centres on work done by Guillino et al., 1956 who have shown that all amino acids are toxic in sufficiently high concentrations and that L-arginine appears to have a protective effect on mice when added to a mixture of otherwise lethal concentration of 9 other amino acids. It is clear that some amino acids are more toxic than others and that the liver and kidneys may well be susceptible to this toxicity. In a preliminary investigation the plasma amino acids were shown to increase in patients on extra corporeal circulation, and therefore this study was partially concerned in more fully elucidating the plasma and urinary amino acid

changes that take place. This is discussed in Section 10 in greater detail. Suffice it say that although the plasma amino acids increased proportionately to the duration of operation, the urinary amino acids did not reach a maximum until 3-4 days post operatively.

Denaturation.

The risk inherent in the use of the extra corporeal circulation is uncertain but morbidity and mortality appear negligible for short bypasses. There is concern however that some of the complications seen after repair of the more complex lesions e.g. aortic/mitral repair or complex atrial/ventricular septal lesions - requiring a longer bypass - may be due to the pump oxygenator itself. Some of these complications may well result from denaturation of blood proteins not only due to the passage of blood through the oxygenator in which it is directly exposed to the gas but also to the use of occlusive rollers in the pump which can at least damage the red blood cells.

Protein denaturation is generally considered to consist in any change in the molecular structure not involving actual alteration of covalent bonds, or at least peptide bonds. A wide variety of chemical and physical agents may bring about denaturation of proteins with its attendant changes of physiological function and biochemical importance. This alteration of protein molecules may be estimated quantitatively by measuring the increase (liberation) of sulfhydryl (-SH) groups which give considerable guidance as to the extent of denatur-

ation of proteins. In plasma albumin the sulfhydryl groups are the most reactive groups in the molecule - imidazole, tyrosyl, and epsilon amino groups of lysine being of somewhat lesser reactivity.

Therefore in this work considerable importance was given to the detection of sulfhydryl groups in both plasma and blood, using different methods and reagents for their assay. The importance of denaturation and sulfhydryl groups has been fully discussed in Section 2.

Various reagents have been used for sulfhydryl assay and these are N-ethyl maleimide (NEM), 5 5' dithiobis - 2 nitrobenzoic acid, (DTNB), p. nitro phenyl disulphide (PNPD), and its mercaptan derivative, p. chloromercuri benzoic acid (PCMB), and its sodium salt, and finally the use of sodium nitroprusside. Although all these reagents were used initially in developing assay procedures for the detection of - SH groups in blood and plasma only two were used finally for the routine examination. These were N-ethyl maleimide and DTNB. Both gave accurate, reliable results and were ideally suited for - SH assay of serial samples in the minimal time. The importance of - SH assay of blood/plasma as soon as possible after collection is stressed in the introduction and methods of Section 2. Storage of samples leads only to complicating factors - such as additional haemolysis and loss (after several days at 3°C) of sulfhydryl content.

Excellent results were obtained with PCMB and PNPD.

In the case of the former, however, standardisation of solutions and need for individual titrations made the - SH assay procedure rather time consuming. The use of PNPD was eventually withheld due to the already satisfactory procedures of NEM and DTNB. DTNB is in fact the water soluble derivative of PNPD and is thus the better reagent to use for the assay of reactive rather than total - SH groups.

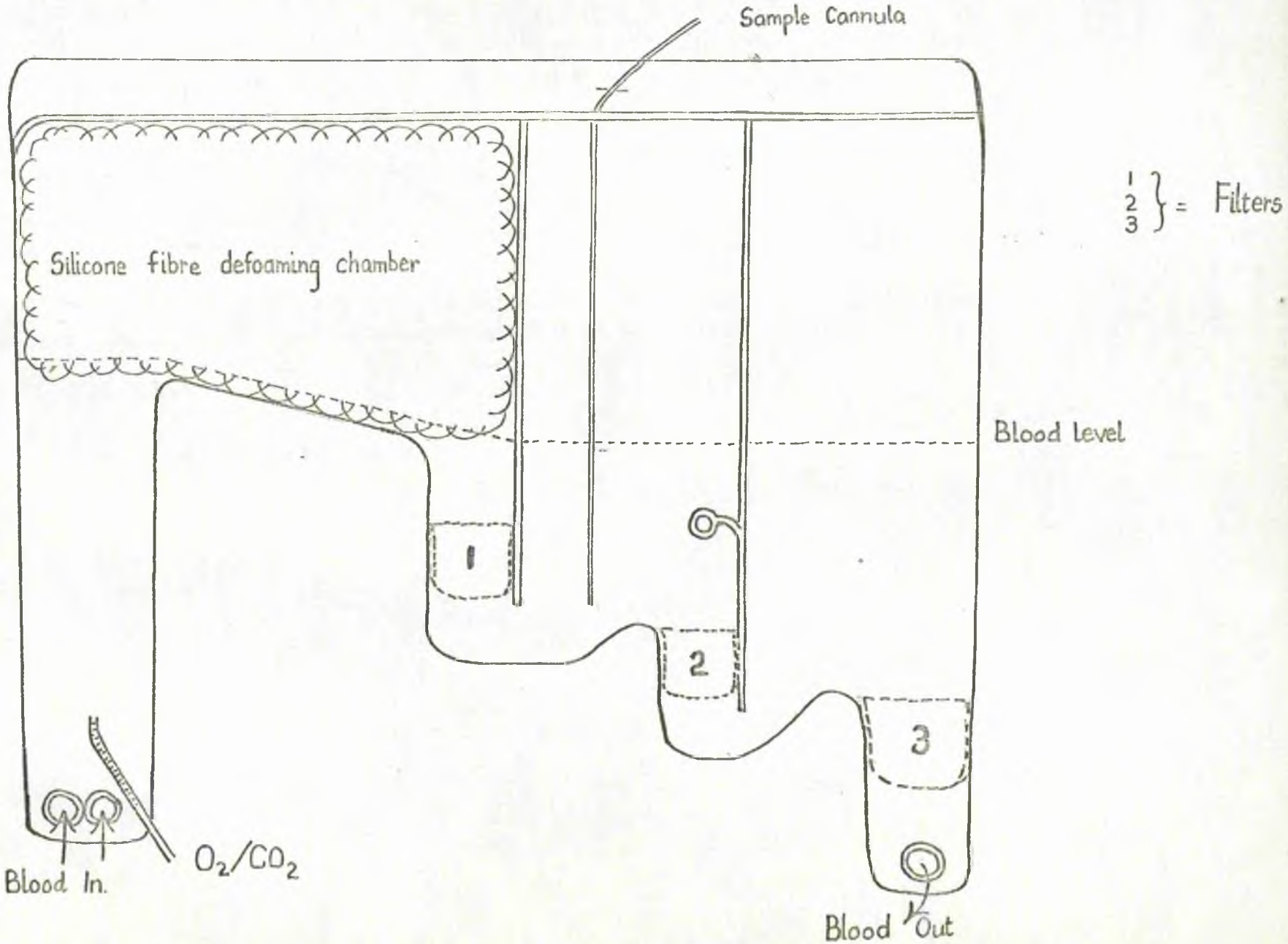
It must be emphasized that the increase in reactive - SH groups is a parameter of mild conditions of denaturation such as those encountered in the blood samples from these open cardiac operations. The total - SH groups of a protein(s) are only obtained after usually severe conditions such as exhaustive hydrolysis with acid or strong concentrated denaturing agents. Apropos of this PNPD was not eventually used for - SH assay because the method uses acetone as solvent - a strong denaturing agent in its own right. Thus the total rather than just the normally reactive - SH groups would be assayed.

Types of Oxygenator

Three types of oxygenators have been used in open cardiac surgery.

1) A disposable 'plastic' Rygg-Kyvsgaard bag Fig. 1.1 and 1.1a. Venous blood enters on the left hand side and is immediately oxygenated by bubbling the gas (95% O_2:5% CO_2) actively through it as it rises to the top of the tube. The blood passes through a silicone wire defoaming chamber and

FIG. 1·1. The Rygg-Kyvsgaard Oxygenator



passes by gravity through three successive filters, which removes any clots and tends to break down any continuing bubbles. It passes out of the bag as the arterial supply.

Blood samples were obtained via the centrally situated cannula. Care was taken in obtaining the initial control samples which were obtained 5-10 minutes after full perfusion had begun. This enabled complete equilibration of the blood perfusate to have taken place before sampling.

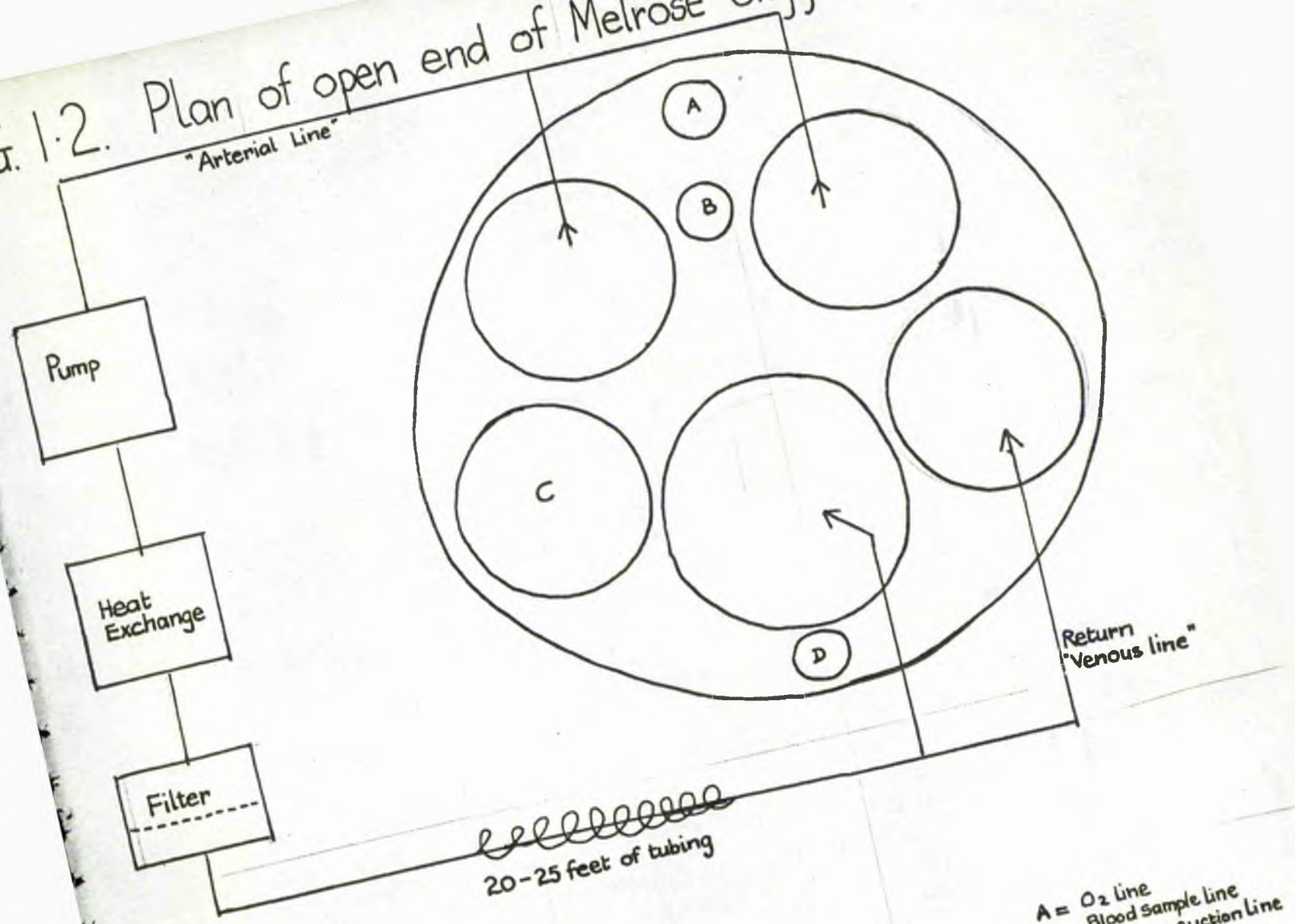
The bag was prepared for use by humidifying and washing through with isotonic saline. The bag was primed by adding three to four pints of blood, one pint of isotonic saline or dextrose saline and usually up to 500 mls of bicarbonate.

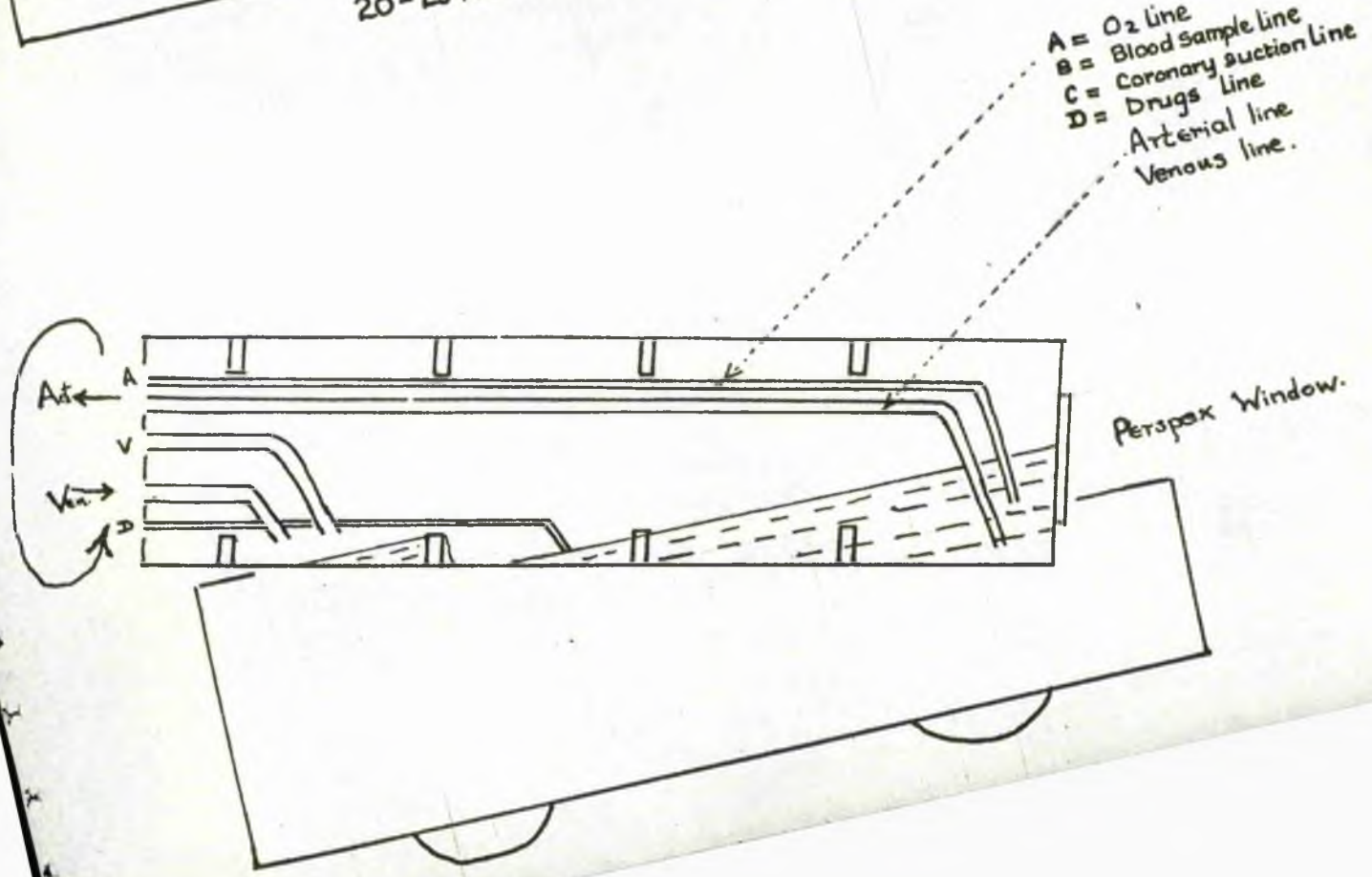
Occasionally the Ryggs bag was joined up with another bag in the circuit (in parallel). This was done in several of the operations which were expected to be of at least 3 hours duration, and gave better oxygenation of the blood.

<u>The Melrose machine.</u> The second type of oxygenator. Fig. 1.2.

This oxygenator takes the form of a cylindrical drum about 36" long and 16" in circumference. The interior of the drum consists of eccentrically placed compartments (four) which prevents the inflowing venous blood from running straight down to the bottom where it pools before being pumped back along the arterial line. The blood thus takes longer to reach the bottom of the drum and thus enables loss of CO_2 and reoxygenation to be facilitated. The drum is placed on the machine at an angle

FIG. 1·2. Plan of open end of Melrose oxygenator drum.





of 20-25° and is rotated throughout oxygenation to allow the venous blood to pass successively through the eccentrically placed compartments. Oxygenation is carried out by bubbling the O_2:CO_2 mixture through the pooled blood at the lower end and allowing the superfluous gas to diffuse gradually up the interior of the revolving drum, as the blood is flowing down in the opposite direction.

It should be added that for the purposes of this study the method and effects of oxygenation of blood by bubbling is similar for both the Ryggs Bag and the Melrose machine, althou in the latter case oxygenation is facilitated by allowing the blood to 'film' around the drum as described above.

Although initially an 'in vitro' experiment (control) was done using the Melrose oxygenator this was at a time when it was in most common usage, however owing to ease of sterilisation and cheapness of disposability, the Ryggs bag is now used in preference, and all the following perfusion studies have been done using the latter.

<u>The Screen Oxygenator.</u> The third type of oxygenator.

This type of oxygenator which was not used in this work, uses the principle of a semi-permeable membrane for gaseous diffusion. Blood is passed one side of a total large area of membrane and O_2:CO_2 mixture the other. Oxygenation is thus obtained by passive diffusion. The efficiency of this type of oxygenator depends ultimately on the total area available for gaseous exchange since there is a limit on the

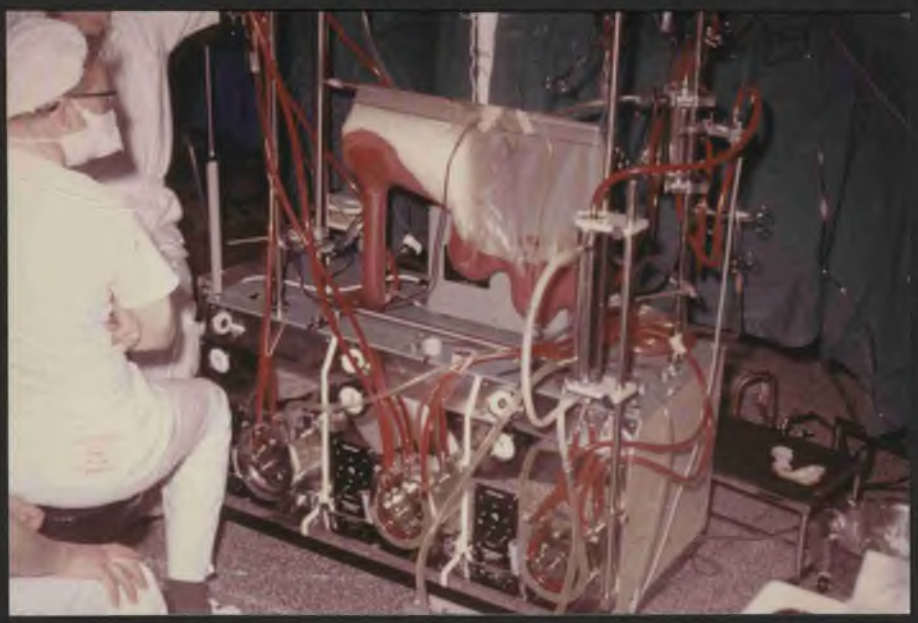


Fig. 1.1a. The Rygg-Kyvsgaard bubble oxygenator bag in use.

Fig. 1.2a. The Melrose Oxygenator.

rate of gaseous exchange per unit area irrespective of the blood flow and rate of oxygen.

Thus the Ryggs bag and Melrose differ from the screen oxygenator in that in the former blood is actively oxygenated by bubbling and filming, whereas in the latter oxygenation is a passive, diffuse process only.

Obviously one of the theoretical dangers of the Ryggs bag and Melrose oxygenators is that micro bubbles may remain in the circulation and get lodged in fine capillaries in the body - the brain and kidneys being of major importance. If this occurs to any extent, it is reasonable to assume that post operative recovery may at least be prolonged as judged by return of consciousness and renal function respectively.

Oxygenating blood by literally bubbling, very actively, oxygen through it, appears a very crude method. It was therefore an interesting as well as a very important practical exercise to assess the degree of possible denaturation occurring in the blood during full perfusion.

Surface action at the interface between a protein solution and a gas or foreign surface is known to produce denaturation. This undesirable tendency to undergo interface denaturation might be minimised by interposing a gas permeable membrane between the blood and gas - it was with this in mind that the membrane type of oxygenator was designed. However, practical results using the Ryggs bag as judged by successful operations and post operative recovery has not necessitated

the use of the screen oxygenator to any great extent in this country although it is employed to a certain extent in the United States and possibly Scandinavia.

The search however for the ideal oxygenator still continues and it is worth while mentioning further, desirable characteristics of the ideal membrane as well as undesirable features to be avoided.

If a membrane is made of hydrophobic (non polar), nonwettable material with a high dielectric constant such as polyethylene, Teflon, or Nylon - then surface polarising forces still tend to denature proteins.

Ideal membranes should be hydrophilic (polar) and of low dielectric constant as well as possessing the other desirable attributes of membranes for oxygenators.

Preliminary work by Wright et al., 1962, has indicated that by using the membrane oxygenator the protein denaturation that took place was indeed only minimal. Other workers, e.g. Lee et al., 1961, came to different conclusions.

Recently, Rubin at the New York City Hospital (Personal Communication) has succeeded in making a membrane of collagen and this could well have the desirable qualities as a membrane oxygenator. By its very nature it can be made hydrophilic or hydrophobic.

The Melrose experiment. Fig. 1.2 and 1.2a.

In preliminary work the Melrose machine was set up under the same conditions as used at operation. Four pints

of blood and one pint of 5% Dextrose was added to the machine. The "arterial" line from the machine passed through in series the pump, the heat exchanger, the filter and back to the machine as the "venous" line. The total length of tubing outside the machine (arterial and venous lines) was of the order of 25-30 feet - this was to simulate the approximate length (and internal surface area) of the extra corporeal tubing at operation.

Blood was circulated at approximately 2½ litres/min. and O_2:CO_2/95:5 bubbled through at 2-2½ litres/min. Blood samples were taken at hourly intervals for 6 hours and assayed for - SH content, viscosity, turbidity, haemolysis and amino acid changes. These results are seen in the respective sections.

The object of this preliminary experiment was to compare the in vitro changes of circulating blood in an enclosed circuit with the in vivo changes of circulating blood from a patient undergoing open heart surgery using the extra corporeal circulation for both oxygenation and maintenance of circulation. This comparison gave interesting results which are included in all the following sections and in the final discussion.

Control operations

The fifteen open cardiac operations using the E.C.C. were compared with two "normal" operations of widely differing duration.

"15 McC" was a man of 46 years who had a hiatus hernia and concomitant oesophageal carcinoma. The operation was remarkable in that it lasted 3 hours and thus afforded an important contrast to the results of 3 hour cardiac operations.

The second operation "17 D.M." was a 65 year old man who had a colonic carcinoma for which he had an anterior resection which lasted 50 minutes.

Other parameters and criteria used in the study.

Although initially studies on plasma protein changes were confined to - SH determination, viscosity, turbidity, haemolysis, amino acid, electrophoretic and ultra centrifugal studies, it became rapidly obvious and desirable to obtain as many other parameters as possible in order to give a more comprehensive and exacting report on blood changes in open cardiac surgery.

The Haemoglobin and PCV (packed cell volume) were taken at frequent intervals not only as a guide to the Hb + PCV blood state but also as a guide 1) to the degree of haemoconcentration/dilution either in presence or absence of added blood or diluent (0.9% isotonic saline, 5% dextrose, dextrose saline or bicarbonate) during the full perfusion. 2) To compare the increasing haemolysis of plasma directly with the Haemoglobin content of the blood at the time of sampling.

The plasma + urinary electrolytes were noted as an indication of fluid balance and to a lesser extent adrenal

function. Obviously these figures were of more importance and interest in cases where little or no blood or diluent was added during full perfusion.

Plasma and urinary creatine concentrations gave some indication of tissue breakdown as also did the plasma uric acid levels.

As an estimation of liver function the SGOT was noted in a few patients.

Haemolysis of plasma increased with duration of perfusion. A method was devised of quantitating this amount of free haemoglobin in plasma by estimating it as cyanomet-haemoglobin. The extent to which plasma is haemolysed is seen in Fig. 5.5. The introduction, methods, results and discussion are seen in Section 5.

The examination of the plasma protein changes were done by electrophoresis on different materials supplemented by ultracentrifugal studies. The results of this work are seen in Sections 5 and 6 respectively.

The other changes which occur in proteins when denatured (or being denatured) are changes in turbidity and viscosity the results of which are seen in Sections 3 and 4. Although optical rotation may be used to determine the extent of denaturation it was not able to be used in these studies.

The separation of protein molecules more or less solely on a molecular size basis was done using Sephadex gel (G 200). This turned out to be a time consuming procedure although

better separations were obtained this way than by using quicker shorter columns. In the separation of a mixture of proteins using one particular gel the method necessarily fractionates some proteins better than others and therefore it is a better practical procedure to either 1) refractionate samples on the same column or use a different gel e.g. DEAE 50 Sephadex - the anion exchanger. The results and discussion of this technique are seen in Section 8.

The cause of stress in open cardiac surgery.

If denaturation of the blood was or appeared to be occurring during perfusion it seemed reasonable that this would add to the stress of the operation and therefore it appeared a good opportunity to measure either plasma ACTH or adrenal cortical activity. Plasma ACTH is very difficult to measure owing to limitation of sensitivity of various methods discussed in Section 9. Adrenal cortical activity therefore was measured by assaying the chief circulating C_{21} adrenocortical hormone - 17 hydroxycorticosterone, - this when reacted (after suitable extraction) with purified phenyl hydrazine yields a yellow chromophore - a phenyl hydrazone which may be estimated quantitatively. Although hormonal assay of perfusion blood has been done by other workers, Walker, et al., 1965, it is greatly possible that owing to improvements in techniques over the last 1-2 years and also because amino acid changes were also being investigated, that the hormonal changes were worth reassessing. It is common practice to add hydrocortisone

(100 mgms hrly) to perfusate for prophylactic reasons (Ghadiadi, Brompton Hospital, Personal communication).

The first 10 cases did in fact have hydrocortisone given during perfusions and case '10 R.M.' was assayed for plasma 17 - hydroxy corticosteroid concentration. In the 3 following perfusions hydrocortisone was purposely withheld - with no ill effects - and the plasma again assayed for 17 - OHCS. Operations 14-17 inclusive also had no additional steroid administered.

Therefore it has been possible to directly compare the amino acid changes in all the perfusions with those (1-10 inclusive) in which supportive adrenal therapy has been given, with those (11-17 inclusive) in which it was withheld, see Section 9.

Feigin et al., 1969 in a preliminary article discusses the circadian periodicity of blood amino acids in normal and adrenalectomised mice. In normal mice the peak amino acid levels are between 0200 - 0800 hours and the lowest levels are seen at 1700 hours - this represents an approximate 56% decrease. The results are given in "total integrated values". The adrenalectomised mice on the other hand, yield blood amino acid levels at lower levels with a maximum around 1700 hours and lowest levels at 2300 hours. These results are virtually the very opposite of these seen in normal mice. The circadian rhythm although altered did not have its periodicity abolished in the adrenalectomised mice. The circadian periodicity of

adrenocortical function was first described by Pincus in 1943.

Corticosterone concentrations in the mouse are at a peak at approximately 1600 hours and at a minimum at 2400 hours. Thus the time of the maxima and minima of corticosterone concentrations in the normal mouse is the same as the amino acid maxima and minima in the adrenalectomised mouse and approximately inversely proportional to the amino acid maxima and minima in the normal mouse.

It would appear from these facts and from the work of Simmons and Nichols, 1966, on the rate of glycine -2-^{14}C uptake in bone cells over 24 hour periods that when the stimulus for protein synthesis is greatest, blood amino acid concentrations is lowest and vice versa.

The results of blood and urine amino acid studies from patients undergoing and recovering from open cardiac surgery have been interesting, - Section 16. It is possible that although the blood amino acids increase during the operation the actual stimulus for new protein synthesis is delayed by factors unknown and that it is not until some hours later when the blood amino acids are decreasing in concentration that 1) stimulus for new protein synthesis occurs and 2) the urinary amino acids begin to increase in concentration.

The interrelationships of plasma corticosteroids, other hormones, and blood amino acid periodicities richly deserves further study.

Drugs used in perfusion.

It is pertinent to mention the few drugs that were used during full perfusion.

1) Heparin was given in initial dose of 3 mgm/Kg and 1 mgm/Kg hourly until the end of perfusion.

The calculation of heparin dosage is simplified thus:-

Weight in lbs x $\frac{5}{11}$ x 3 x $\frac{13}{10}$ = dose in mls of a solution containing 1000 I.U./ml.

2) E.A.C.A. - epsilan amino caproic acid was given in many cases in concentrations of 4 Gm stat. followed by 1 Gm hourly. This drug - recently innovated - is used prophylactically in conditions where there is increased fibrinolysis (fibrinogenolysis) with haemorrhage or risk of haemorrhage.

3) Mannitol 20% solution was used in many cases to facilitate urine flow during perfusion - which hitherto was often absent or of small volume e.g. 10-20 mls. 40 mls was given at the commencement of perfusion and at hourly intervals.

4) The use of Hydrocortisone has been commented on above and is discussed in greater detail in Section 14.

5) Sodium bicarbonate 4.2% was used initially in the starting perfusate volume, as below.

6) Potassium was given as KCl in some cases. 1 gm being added in the priming perfusate volume and another gram on the appearance of urine. This

additional potassium was to replace that lost in the urine due to the mannitol administration. Needless to say the blood electrolytes were screened half hourly to maintain electrolyte balance.

The priming perfusate volume (P.P.V.)

The total perfusate volume depended on the normal blood volume of the patient and apropos of this - the surface area (rather than the body weight) of the patient. As a general rule in most cases the P.P.V. consisted of the following

1) 3-4 pints of whole blood.
2) 1-2 pints of 5% dextrose/saline.
3) 150 mls of 4.2% sodium bicarbonate.
4) ? plus mannitol, potassium chloride, hydrocortisone etc.

Design of Thesis.

This investigation into plasma protein and amino acid changes which take place during open cardiac surgery has been shown in the following form for 1) ease of reference and 2) because some sections are clearly unrelated to others.

Section II concerns denaturation, the methods of measurement with especial emphasis on sulfhydryl (- SH) groups.

Sections II A, B, C, D and E include the sulfhydryl investigation of denaturation using agents N.E.M, D.T.N.B, PCMB, PNPD and sodium nitroprusside respectively.

Section III changes in turbidity of a protein in an 'in vitro' system are also a parameter of denaturation but as has been seen with viscosity, changes take place in blood during perfusion which rather suggest that denaturation is only one of the factors - and possibly least important - responsible for changes (increase) in both viscosity and turbidity. In the latter case there is evidence that stress affects the blood lipaemic picture. The significance of turbidity changes in open cardiac surgery is therefore discussed in a separate section.

Section IV. Although denaturation of a pure protein may be investigated by the use of viscosity changes it has been found that in the in vivo 'state of flux' environment of open cardiac surgery - viscosity changes cannot be used as an absolute parameter of denaturation owing to such specific factors as haematocrit change, haemolytic effect and the release of unknown toxins or proteins into the blood ? as a result of stress. Plasma viscosity changes are therefore discussed in a separate section where an interpretation of the results is given.

Section V. The occurrence of marked haemolysis during perfusion has given rise to the possibility of its participation in the increase of blood amino acids, and although not mentioned before - its participation in blood sulfhydryl group increase and not unreasonably viscosity and turbidity.

Section VI. This section deals with a variety of electro-

phoretic methods which were used in initial plasma protein separation on different media.

Section VII. Ultracentrifugal studies: Although these studies also deal with plasma protein separation, the emphasis of changes, if any, is on the molecular weights of the major protein constituents of plasma at the beginning and end of full perfusion.

Section VIII. Column chromatography using G 200 was used in the separation of plasma proteins according more to molecular size rather than net charge (as in electrophoresis). The method, results and discussion using this technique are seen in this section.

Section IX. The importance of hormonal studies has been raised earlier in this introduction. In this section the difficulty of ACTH assay is described together with the 17 - OHCS plasma estimations from various operations. The importance of these findings together with the amino acid investigation of the next section is discussed in the latter section.

Section X. The amino acid changes of plasma during perfusion, and urine in the post operative period are investigated and the cause of such changes discussed in relation to haemolytic changes, denaturation of plasma proteins, hormonal factors and hepatic metabolism.

The final discussion is given in which the sulfhydryl assay of plasma, turbidity, viscosity, haemolytic, electro-

phoretic, ultracentrifugal analysis, column chromatography, haemolytic, hormonal and amino acid results are discussed as a whole.

A final diagram is proposed to explain the changes found during and following these perfusion operations.

SECTION 2.

DENATURATION Introduction.

Denaturation is a transconformational reaction of proteins. Kauzmann, 1959, widely defines this unique protein change as a process (or sequence of processes) in which the spatial arrangements of the polypeptide chains within the molecule is changed from that typical of the native protein to a more disordered arrangement. Although this is a broad, unspecific definition it still does not include all transconformational changes.

Simply it is a loss or alteration of the secondary + tertiary structure of a protein(s). It does not involve hydrolysis of peptide linkages but the chemical characteristics of denatured proteins is different from the native protein.

This elusive problem is best tackled by mentioning

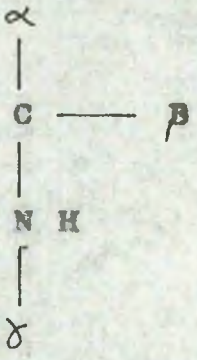
1) The causes of denaturation
2) Characteristics of the denatured protein
3) Methods of measuring the rate or absolute change of denaturation -
4) Significance of denaturation.

It must be emphasized that the results of denaturation obtained by a specific method can only be compared and utilised with other data using the same method.

CAUSES OF DENATURATION

I Chemical Denaturation

Many effective denaturants have structures similar to urea. So that the following generic structure may be used to illustrate this.



α = $-NH_2$

β = =O

γ = H

S - Methyl isothio urea also follows this structure $\beta = NH_2$, $\alpha = CH_3S$ $\gamma =$ H.

Guanidinium salts - ($\alpha =$ NH_2, β = NH_2 NH.HCl and $\gamma =$ H).

As usual there are many exceptions to this formula and one must mention similar but nonetheless different chemicals. The vast number of solvents are good denaturants.

Acetone $(CH_3)_2$ C = O

Ethanol $CH_3.CH_2.OH$

Dimethylformamide $(CH_3)_2$ N. CO. H.

In all of them one can still see however (a) oxygen function in the form of carbonyl or hydroxyl groups and (b) dimethyl or substituted methyl groups.

Inorganic metals or metallic salts will also cause denaturation e.g. Li Br, "$(NH_4)_2SO_4$", + sodium salicylate.

The long chain alkyl sulphates (antifoaming, wetting agent) e.g. Sodium dodecyl sulphate

$CH_3\ (CH_2)_{11}\text{-}O\text{-}SO_3.Na$

is also an active denaturant, being effective in very dilute concentrations. Acids + alkalis are also good denaturants.

Chemically denaturation results in an increase in detectable (reactive) sulfhydryl and disulphide group. The latter of which are detectable after reduction to - SH. Some proteins have detectable - SH groups but no apparent - S - S - groups - as reflected in no further increase in - SH groups after the possible - S - S - bonds have been reduced.

This detection becomes a little more complex when it is remembered that not all sulfhydryl groups are reactive and that there are often latent or hidden sulfhydryl groups which either totally or only partially become expressed during the process of complete or incomplete denaturation.

It must be emphasised that seemingly complete denaturation may still not release all the - sulfhydryl or disulphide bonds present in the molecule.

Paradoxically - must one assume that complete denaturation has taken place if there are no more - SH or - S - S groups present (as done on other aliquots by exhaustive chemical analysis).

To complicate the issue still further, - in the sulfhydryl analyses of biological fluids (blood, plasma) it is difficult to know whether the - SH groups detected are due or partially due to small molecules e.g. glutathiane, ergothioneine, cysteine etc. and how much due to proteins and their denatured relatives.

It is important to appreciate that the sum total of detectable sulfhydryl groups - however obtained is also partly due to:-

1) the reagent employed
2) the activity of the sulfhydryl groups for both the reagent and reaction conditions (e.g. temp, oxygen presence etc.)
3) the chemistry and stoichiometry of the reaction
4) the specificity for sulfhydryl groups
5) selectivity for sulfhydryl groups of different reactivity
6) the sensitivity and accuracy

and lastly

7) the presence of turbid or coloured solutions and the presence of denaturing agents (interference).

Chemical denaturation of plasma albumin in the presence of concentrated urea solutions shows interesting changes, optical rotation and viscosity both increase and further changes result in gel formation. The first rapid changes are reversible but the later secondary changes suggest that a disulphide interchange reaction takes place with the formation of intermolecular disulphide bridges. Evidence of Huggins, 1951, indicates that sulfhydryl groups are essential for these secondary changes.

$$R - SH + S - R_1 - S \longrightarrow R - S - S - R_1 - SH$$

Where R_1 and R_2 are two protein molecules. An

intramolecular disulphide linkage is thus replaced by an intermolecular linkage. This is in fact a type of chain reaction for the new - SH group is free to react again with the eventual formation of a gel. It is interesting that only catalytic amounts of - SH are necessary for this reaction.

In haemolysed plasma for instance not only is the haemoglobin available for denaturation and consequently increased - SH detection, but also the glutathione is freed into the plasma (normal mean concentration 35 mgm/100 ml).

In conclusion therefore sulfhydryl detection in blood or plasma from operations represents the sum total of available reactive groups and may be summarised thus:

The rate of increase in - SH groups due to denaturation (and haemolysis) must be in excess of the rate of loss due to

1) Changes in dilution
2) Storage
3) Oxidation
4) ? Reversibility of denaturation
5) Haemoconcentration* (reduces denaturation rate)
6) return of pH to native protein pH
7) unknown (? multiple) factors.

*As mentioned in the physical causes of denaturation, dilution of a protein favours denaturation.

Bearing in mind the above factors it is not surprising that different methods of sulfhydryl determination (chemical assays) do give different and sometimes apparently unrelated data.

I(b) Physical causes of denaturation

These include heat, ultraviolet irradication, X-rays, violent shaking, and extremes of pH.

From the point of view of investigating the degree of denaturation taking place in plasma proteins during cardio pulmonary bypass the above fact of Violent shaking requires mention and clarification.

In both the Rygge Bag and Melrose oxygenator the blood is oxygenated by bubbling O_2 (95% O_2:5% CO_2) through at rates of 3-6 litres/min. according to Blood Volume (Total); - the latter of which is the sum of the blood volume of the patient and the volume needed for priming the extra corporeal circulation.

The action of bubbling O_2 through the blood is identical with shaking it, and hence the question of denaturation. The occurrence of haemolysis and its importance is mentioned elsewhere.

Dilution of a protein solution also favours denaturation - this fortunately has turned out to be a negligible problem - contrary to first impressions. Since not only in some operations was no dilution occurring but plasma proteins would have to be considerably diluted before denaturation solely due to this cause occurred.

A protein is most stable against denaturing action at the pH of its native state. Since the pH of blood varies so very little during perfusion (e.g. 7.47-7.51) this additional

feasible cause of plasma protein denaturation is negligible.

2 Characteristics of the denatured protein

By use of x-ray diffraction it has been shown that globular proteins e.g. albumin and globulins which in the native state are in an ordered helical configuration assume a progressively more random state (random coil formation). This is brought about by rupture of sulfhydryl and disulphide bonds, which in turn lead to unfolding or uncoiling of the constituent peptide chains.

Whether this change is linear or stepwise in form has been mentioned in the work on sulfhydryl analyses.

With an increase in denaturation, viscosity increases, and also the liberation of sulphydryl, disulphide, and tyrosine groups occurs and most probably other groups that have hitherto not been detected. The disulphide groups are detected by first reduction to sulfhydryl. The appearance of the latter groups in denaturation is not due to their formation directly from disulphide bonds since they exist in their own right intramolecularly.

At the isoelectric point where a particular native protein is soluble, the denatured protein becomes insoluble.

In the denatured state enzymes and protein hormones are inactivated. Haemoglobin loses its colour and no longer allows oxygen to associate.

Some proteins in the presence of certain denaturing agents show the phenomenon of reversible denaturation.

Anson and Mirsky (1925 and 1931) demonstrated the

reversibility of protein denaturation by using Haemoglobin. This was denatured by acid and then shortly after exactly neutralised to dissolve the precipitated Haemoglobin. He found that 66% remained in solution and showed characteristics of the native protein i.e. absorption data and oxygen affinity. This recoiling of the peptide chain is seen with globin and albumin when the pH is readjusted to that of the native protein.

Reversible denaturation can also be shown to occur with Haemoglobin in the presence of neutral sodium salicylate. This denatured Haemoglobin is digested by trypsin; but if the salicylate is removed the protein reverts to its native state in which it is not digested by trypsin.

It is worthy of comment that all the first few proteins which have been shown to exhibit reversible denaturation have been those of blood.

A denatured protein is oxidised if it gives no colour reaction with nitroprusside.

Since a percentage of lipid in the blood is present normally in the form of lipoprotein (α + β globulins), it is possible that if any significant denaturation takes place in operations using cardiopulmonary bypass, the lipid may be freed from its normal complex and be responsible for micro fat emboli in the blood.

Secondly, minor damage to the red blood corpuscle - i.e. before haemolysis commences - may lead to intravascular haemagglutination or sludging. The action of the pump

rollers on the oxygenator may well initiate the latter, thus being responsible for pre-haemolytic as well as haemolytic changes.

It is of interest that native egg albumin contains no reactive sulfhydryl groups but that if denatured, the number of sulphydryl groups detected is equal to the number found after complete hydrolysis with acid. The degree of denaturation may be followed by the number of free or reactive sulphydryl groups.

It has been shown that the rate of denaturation is

1) Independent of pH

2) A function of a particular protein.

Simko and Kauzmann (1962) in work on haemoglobin denaturation by urea has shown that the rates are roughly fifth order. Under identical denaturing conditions the related haemoglobins - cyanhaemoglobin, methaemoglobin and oxyhaemoglobin differed by more than two orders of magnitude. This means that there are more than two phases in the denaturation process which are proceeding at rates slower than another phase, and which are governing the rate of denaturation.

III Method of measuring the rate or absolute change of denaturation.

Much has been mentioned about the importance of sulphydryl groups already and thus the following reagents have been utilised in the succeeding work.

1) N - Ethyl maleimide
2) 5.5' dithio bis 2 - nitro benzoic acid
3) p. chloromercuri benzoate
4) bis p. nitrophenyl disulphide
5) sodium nitroprusside.

Amperometric (polarographic) determination of sulphydryl groups is mentioned in the PCMB section.

As sulphydryl groups are easier to determine than disulphide (Cavallini et al., 1966), used sodium boronhydride ($NaBH_4$) (as reducing agent) and 8M Urea to assay sulphydryl groups in protein.

Alkylation procedures.

Two other methods are worth mentioning.

1) The reaction of protein with iodoacetate and sodium lauryl sulphate to form carboxy-methylated protein, which after hydrolysis is assayed directly as S- carboxymethylcysteine, by partition chromatography. This measures total sulfhydryl only, Spackmann et al., 1958.
2) Iodoacetamide reacts with sulphydryl groups with a release of hydrogen ions. The pH of the reaction is kept absolutely constant by the

addition of NaOH. This method is ideal for determining sulphydryl content as well as the rate of the reaction. A sensitive pH stat is essential. The end point is the stage when no more NaOH is required to keep the pH constant. The volume of caustic added is directly related to the number of - SH groups present.

The turbidity of a protein increases with denaturation and thus this simple technique was used by noting the absorption change at 620 mμ. The patients from whom the samples were taken were all in the fasted state and the volume of blood used to prime the extra corporeal circulation would have been considerably diluted by the patients own volume; hence any overt transient hyperlipaemia due to donor blood would have been diluted and indeed well mixed before the control sample was taken, - of this more later.

Viscosity of a protein increases with its denaturation and this fact was utilised in assessing the degree of denaturation taking place in plasma (undiluted) samples taken at serial (half hourly) intervals during extra corporeal bypass. As stressed in the viscosity section however other factors occurring during perfusion make these changes unreliable as a direct parameter of perfusion denaturation.

Other <u>hydrodynamic properties</u> which may be used are:

1) the frictional ratio

2) the rotatory diffusion constant.

Light scattering and small angle X-ray scattering are forms of radiation scattering which may be used. For logistic reasons the former could not be used in plasma changes.

Thermodynamic properties of a denatured protein may be investigated by 1) energy and heat capacity and

2) solubility of the protein.

Optical changes

Gordon and Jencks, 1963, using optical rotation at 579 mu studied denaturation of protein bovine serum albumin using 163 denaturing compounds.

Other optical methods are:

Infrared absorption and dichroism, and the much used ultraviolet and visible absorption.

Lastly, the index of refraction may be used.

Other Chemical methods

Immunochemical techniques e.g. double diffusion technique of Ouchterlony, 1958, using agar gel may be used. Wright et al., 1962, using this method employed anti canine globulin rabbit serum as reagent to compare the precipitation bonds formed (at the junction of antigen antibody) in plasma controls and plasma which had been circulated for several hours through membrane and screen oxygenators.

Other chemical methods include biological activity of denatured protein and electrophoresis. The latter was used on different media - paper, starch, cellulose acetate and acrylamide gel. A section will be devoted to this, (6).

IV The Significance of Denaturation

There has been concern that prolonged cardiac bypass increases both morbidity and mortality of these operations. It has therefore been pertinent to examine the plasma proteins to assess the degree of denaturation and its subsequent relation, if any, to operative stress and post operative recovery.

'Damaged' proteins may interfere in the following manner:

1) They may exacerbate the inflammatory response - due to alteration of structure and therefore looked upon as foreign protein by the body.
2) Cleavage of certain groups on a protein molecule could increase the plasma amino acid content to a variable extent dependent on severity.
3) Damaged proteins are more easily precipitated in small vessels with micro emboli (O_2:CO_2) from the oxygenation site, this enhances poor circulation and sludging of cellular components of blood.
4) The association of damaged blood proteins and stress is difficult to relate in quantitative terms, but it is nonetheless extremely important.

Introduction to sulfhydryl analyses

As pointed out in the previous section on denaturation there are many reagents which may be used to assess the degree

of denaturation and the absolute increase in reactive only and in some cases total sulfhydryl groups.

This section will deal simply with introducing the reagents used in assessing the degree of denaturation of plasma proteins in open cardiac surgery, and mention other methods, which could be used but some of which are accompanied by practical disadvantages and difficulties.

The following reagents were used in the following practical work, and the reader is referred to them for the appropriate details.

1) N - Ethylmaleimide (NEM)
2) 5.5' dithio bis 2-Nitro benzoic Acid (DTNB)
3) p. chloromercuri benzoic Acid and Sodium salt (PCMB)
4) p. nitrophenyl disulphide (PNPD)
5) Sodium nitroprusside (NaN.P)

Various workers - starting with Kolthoff and Harris in 1946 have used the polarographic technique for estimating sulfhydryl groups. This is mentioned in the PCMB section.

The use of polarography has not been used extensively in quantitative sulphydryl investigations of proteins for various reasons.

1) The absolute necessity for oxygen removal and maintenance of an oxygen free environment.
2) The virtual absence of suitable, accurate, reliable equipment which is not affected by neighbouring apparatus.

3) Poor familiarity of the complex polarographic techniques. (The Introduction to Polarography by Milner contains 1200 pages).

Benesch, R. and Benesch R. (1950) have used the amperometric technique in the determination of soluble mercapto groups in blood and tissues.

Woodward, et al., (1932), Quensel, et al., 1935 Wacholder, et al., 1935, Fujita, et al., 1938, Schroeder, et al., 1939, Binet, et al., 1934 have in their determination of glutathione sulfhydryl used iodine as oxidising agent in the titration of tissue filtrate.

There are two main disadvantages to this technique -

1) Lack of specificity - due to interference by ascorbic acid.

2) The dilution effect. The result depending on the I_2/GSH ratio owing to oxidation passing beyond the disulphide stage.

Due to metaphosphoric acid and acid alkali (protein precipitants) destroying the glutathione present, - 25% sulphosalicylic acid is used, as glutathione is stable in the latter.

EDTA (Ethylenediamine tetraacetic acid) 3×10^{-5} M was used to prevent (by chelation) the metal catalysed oxidation of glutathione in the alkaline titration mixture.

Ascorbic acid did not interfere in this assay. Unfortunately the sulfhydryl detected was of both glutathione

and - to a lesser extent - ergothionine.

Anson in 1940 and 1942 and Hess and Sullivan - 1943 also estimated the sulfhydryl groups of egg albumin by iodine titration.

Alkylating agents may be used to assay sulfhydryl groups but only under properly controlled conditions, since they otherwise react with imidazole, amine groups and methionine. Iodoacetate and iodoacetamide are most commonly used.

Total sulfhydryl groups of protein may be assayed at pH 9 by reacting with excess iodoacetate in the presence of sodium lauryl sulphate (denaturing agent). - SH is assayed as S - carboxymethyl cysteine, according to Spackman et al., 1958.

The reaction of sulfhydryl groups of protein with iodoacetamide can be followed by the concomitant liberation of hydrogen ions. Thus both the rate of the reaction as well as the sulfhydryl concentration may be found. The H^+ ions liberated are measured at constant pH between pH 7.0 - 7.5 (critical) during the addition of 0.01 N NaOH. The latter is only added to keep the pH constant. The maximum volume added is equivalent directly with the number of - SH groups present. This is the method of Fraenkel - Conrat et al., 1951. It is not known whether this method detects total or just reactive - SH groups.

Recently Wronski, 1967, has described a submicro method for the determination of thiols, disulphides and thiol

esters in serum by using O - hydroxymercuri benzoic acid (HMB) and dithiofluorescein (DTF). The reaction is done in alkaline solution with both HMB and DTF in excess and the extinction measured at 588 mμ. It is interesting that Wronski has found an unknown substance X in serum which produces green complexes with D.T.F. and undergoes decomposition on addition of formaldehyde. This substance X is eliminated in serum estimations by a separate assay.

The problems of differentiation between protein and non protein sulfhydryl groups mentioned elsewhere in this work is of considerable importance in the elucidation of the cause of increased sulfhydryl groups of blood and plasma observed in samples from open cardiac surgery. Jocelyn has attempted this differentiation by estimating protein and non protein (glutathione) sulfhydryl groups in the presence of each other using the reagent di- (5 carboxy - 4 - nitrophenyl) disulphide, which was first shown to react with proteins by Ellman, in 1959, although not specifically with sulfhydryl groups at this time. Jocelyn has measured at 412 mμ the - SH concentration of glutathione at pH 6.8 and the - SH concentration of serum albumin at pH 7.6.

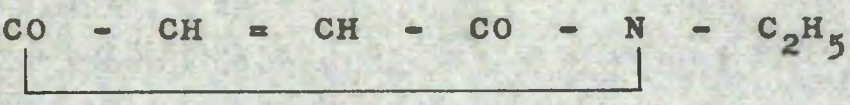
In order that the total sulfhydryl concentration of proteins, filtrate or tissues may be obtained, relatively drastic procedures are needed which take into no account the natural reactivity of sulfhydryl groups.

Normally reactive groups are made available under as mild conditions as possible. This was the object in sulfhydryl detection in the blood/plasma specimens obtained from patients during open cardiac surgery.

The unreactive sulfhydryl groups can only be obtained by denaturation - using unnatural chemical or physical means which are moderate to drastic procedures.

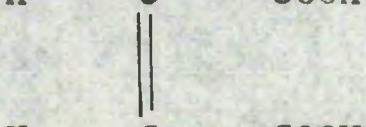
Section 2A.

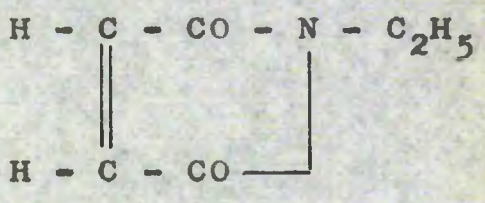
N-Ethyl Maleimide (N.E.M.)



Introduction

Sulfhydryl compounds in addition to reacting with carbonyl groups to form thiohemiacetal and thiohemiketals, also react with activated double bonds. This latter fact is made use of in analytical biochemistry in reactions with compounds derived from maleic acid.

Maleic acid 

N - Ethyl maleimide 

N-ethyl maleimide has gained prime importance due to this fact.

The usual reaction is carried out in 0.1M̄ phosphate buffer at pH 6-7 since at higher pH's significant hydrolysis of the maleimide takes place and this is responsible for the loss of absorption at 305 mμ.

Below pH 6 the reaction with thiols is too slow.

N-ethyl maleimide has proved to be a most useful reagent for sulfhydryl estimations for four reasons:-

1) The technique is simple and relatively quick and therefore

2) Ideal in routine testing of serial samples.

3) No standard thiol solutions (e.g. glutathione) are needed for calibration.

4) No Nitroprusside is needed as an external indicator.

like the majority of techniques it has its disadvantages which are:

1) The Molar Extinction Coefficient is only 620/M/cm

2) Compared with p.chloromercuri benzoate (PCMB) it only reacts with about 60% of the sulfhydryl groups in the protein under test. This needs some clarification:

It may well be that N-Ethylmaleimide reacts with all the available or reactive sulfhydryl groups which are present but that PCMB reacts with a further 66% which are of a more unreactive nature. The importance of this phenomena depends solely on the type of protein in question. Different proteins giving widely differing and sometimes very confusing results.

3) Care must be used when using NEM since it is a powerful vesicant.

Principle of Method and versatility of use.

The change (decrease) in absorption at 300 mμ is directly proportional to the concentration of sulphydryl groups present - when N-ethyl maleimide is present in excess of 10%.

Many other workers have used this compound for a wide variety of purposes since Alexander described its merits in 1958.

Benesch, 1956, extended the use of N-Ethylmaleimide by detecting thiols and thiol esters in paper chromatography.

Ingram, 1955, has used N-ethylmaleimide in blocking the thiol groups of ox and horse haemoglobin but not stoichiometrically. At neutral pH and room temperature two moles of maleimide blocked only 1.5 out of the 4 sulphydryl groups available in horse Haemoglobin.

Friedman et al., 1949, and Marrian in 1949 showed that N-ethyl maleimide readily reacts with such compounds as glutathione and mercapto acetic acid.

Tsao and Bailey in 1953 used N-ethyl maleimide to block all the sulfhydryl groups of actin and myosin, stoichiometrically. These proteins were previously denatured with guanidine hydrochloride, as the maleimide only reacted with 40% of the sulfhydryl groups in native myosin.

Katz and Maxwell, 1964, used both maleimide and PCMB in the determination of sulfhydryl groups in actin prepared from both cardiac and skeletal muscle sources. It is interesting that N-ethyl maleimide reacted with all six sulfhydryl groups (per 60,000 gms protein), like PCMB.

Using radioactive maleimide Riggs and Wells, 1960, showed that the reactive sulfhydryl group in Haemoglobin A is in the β chain. The single and equivalent sulfhydryl group in the γ chain is likewise reactive but the sulfhydryl of α^{F} chain is not.

Again in regard to the binding of Haemoglobin and oxygen Benesch and Benesch, 1961, have indicated that the view is no longer held that the oxygen linked acid groups are sulfhydryl since the latter can be blocked by N-ethyl maleimide without altering the Bohr effect - that is the dependence of oxygen affinity upon acidity.

Because of the low molar extinction coefficient difficulties are encountered when relatively high protein concentrations are required, - when the sulfhydryl content is low. This can result in blank absorptions in the 300-305 mμ range which are too high for accurate assay.

Cole et al., 1958, ran into this problem and therefore when using N-ethyl maleimide determined the sulfhydryl groups of globin rather than Haemoglobin.

Materials.

Phosphate buffer 0.1$\bar{M}$, pH 6.8

Fresh solution of N-ethyl maleimide. 0.0313 gm made up to 250 mls in buffer gives a 10^{-3} $\bar{M}$ solution. This solution reacts with sulfhydryl groups in the concentration range of 9.0×10^{-4} $\bar{M}$ $\longrightarrow$ 1.0×10^{-4} $\bar{M}$.

N-ethyl maleimide slowly decomposes in 0.1 $\bar{M}$ phosphate buffer. Keeping the solution at 2-3°C for 5-7 days is the recommended maximum. After this time it is necessary to make up a new solution.

A fresh NEM solution has an absorption of 0.62 (1.0×10^{-3} $\bar{M}$) at 300 mμ. After 1 week the absorption has fallen to

around 0.533 at 300 mμ and 0.525 at 305 mμ.

Method.

To each estimation three tubes (5-10 ml volume) are required.

1) The reactant tube contains unknown (plasma), N.E.M. and buffer.
2) The unreactive tube contains Maleimide (NEM) and buffer only. As this tube had no variables it was used for each estimation.
3) The blank, contains plasma and buffer only.

In this way the difference in absorption of maleimide/buffer in tube (2) and maleimide, buffer and plasma tube (1) is solely due to sulfhydryl concentrations.

At least 2-3 estimations should be done per sample, preferably by varying the substrate and/or NEM concentrations by a small factor.

The reaction of maleimide with sulfhydryl is immediate and the absorption of Reactive and unreactive tubes may be read after thorough but gentle mixing.

NEM does not absorb light at 300 mμ. The difference in absorption between reactant and unreacted solutions is divided by the Molar Extinction Coefficient of 620. The resultant is the molar sulfhydryl concentration of the sample.

As the molar extinction coefficient is low, relatively high protein concentrations are needed especially if the sulfhydryl content of the protein is low. The difficulty of

blank absorptions in these cases may be overcome by precipitating the reaction mixtures with perchloric acid at a final concentration of 2% and determining the extinction at 305 mμ in the filtrate. A filtrate similarly prepared without the presence of N. Ethyl maleimide acts as the blank.

A 1% solution with one reactive sulfhydryl group per 100,000 molecular weight would be 0.0001 Molar with respect to the sulfhydryl concentration, which is within the limits of sensitivity of the method.

In most of the following work maleimide was used for sulfhydryl estimations of plasma only, although the reagent may be used with equal success on blood.

RESULTS.

Calibration 2A.I. Optimal concentration of maleimide.

Keeping the plasma volume constant the volume of maleimide was varied. Keeping the total volume of reactants constant was met by

$$\text{Volume of NEM in buffer} \times \frac{1}{\alpha} \text{ Volume of buffer.} = k.$$

where k is a constant of 10 mls.

From fig. 2A.I it is seen that as soon as the maleimide volume is in excess of 30% of that of the total volume of reactants, - then maximal absorption is reached, and does not decrease with continued increase in maleimide volume. 0.05 mls plasma was used, and the buffer as mentioned before was 0.1 $\bar{M}$, pH 6.8.

Calibration 2A.2. Minimal concentration of maleimide.

In the second experiment maleimide was reacted with serial increases of glutathione (reduced) 0.0005 $\bar{M}$ such that

$$\text{Volume of NEM} \times \frac{1}{\alpha} \text{ Volume glutathione} = k$$

The total volume of reactants was again 10 mls.

It is clearly seen that linearity is observed with increasing glutathione concentration until the maleimide volume has decreased to 4 mls 0.001 $\bar{M}$, when there is an abrupt fall. This indicates well that at this point there is insufficient maleimide for the concentration of sulfhydryl groups present.

It also shows that providing NEM is in excess of 30-40% volume - bearing in mind the concentrations of reactants in

FIG. 2A·1 Optimal concentration of Maleimide.
(Plasma 0·05 mls.)

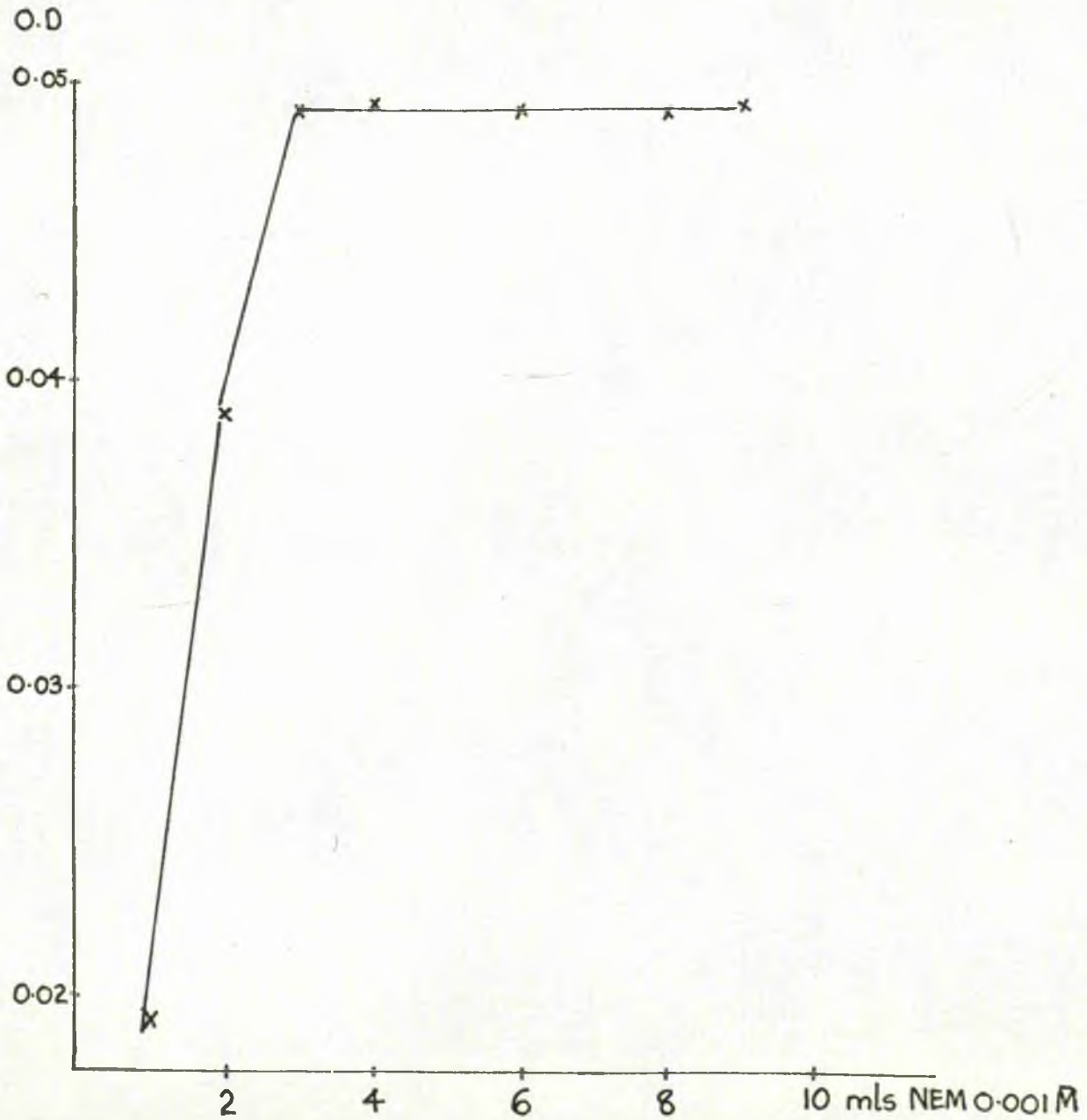
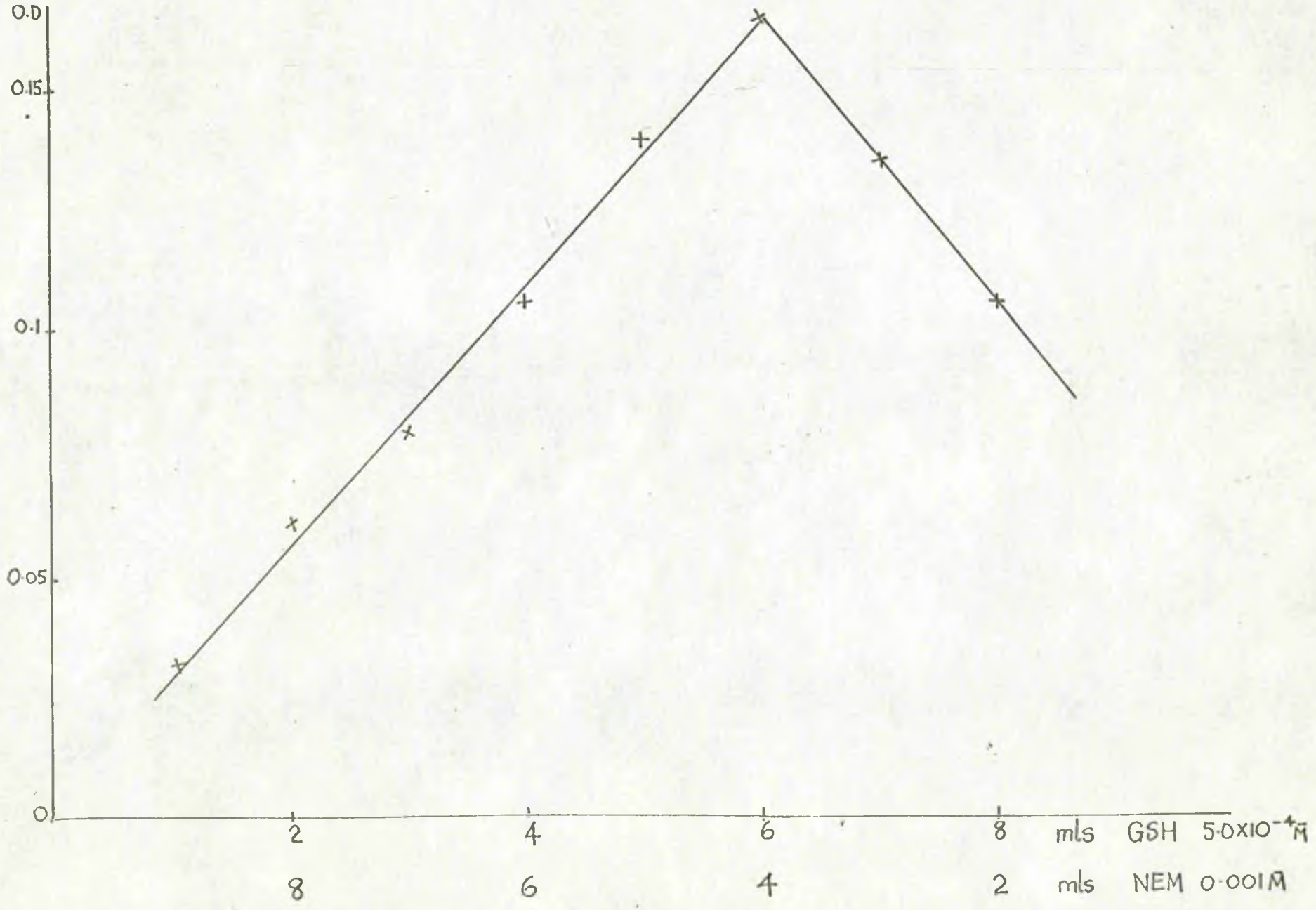


FIG. 2A·2 Minimal concentration of Maleimide.



these experiments, - then Beers Law is observed. This is an important conclusion.

Glutathione calibration: Fig. 2A-3.

Keeping the maleimide volume constant (9 mls), the reagent was reacted with serial increases of glutathione (0.0005 $\bar{M}$). Strict linearity is seen.

It appeared therefore that the criteria of volume and concentration in this sulfhydryl analysis of plasma would satisfy Beers Law by

1) Using maleimide in as great a volume as possible and
2) due to 1) any great increase in the sulfhydryl concentration of plasma would still give consistent and accurate results.

Therefore for most future analyses the following volumes and concentrations were used.

NEM	4.5 mls	1.0×10^{-3} $\bar{M}$
Plasma	0.05 mls	1.274×10^{-4} $\bar{M}$ (Normal Mean Value)
Buffer	0.45 mls	1.0×10^{-1} $\bar{M}$ pH 6.8.

The importance of using as high a concentration of maleimide as possible means that the control values themselves will be higher, and therefore more sulfhydryl groups can be detected before zero optical density is reached.

Emphasis is laid on the fact that increase in sulfhydryl concentration leads to a decrease in optical density after reaction with maleimide.

FIG 2A·3 Glutathione calibration.

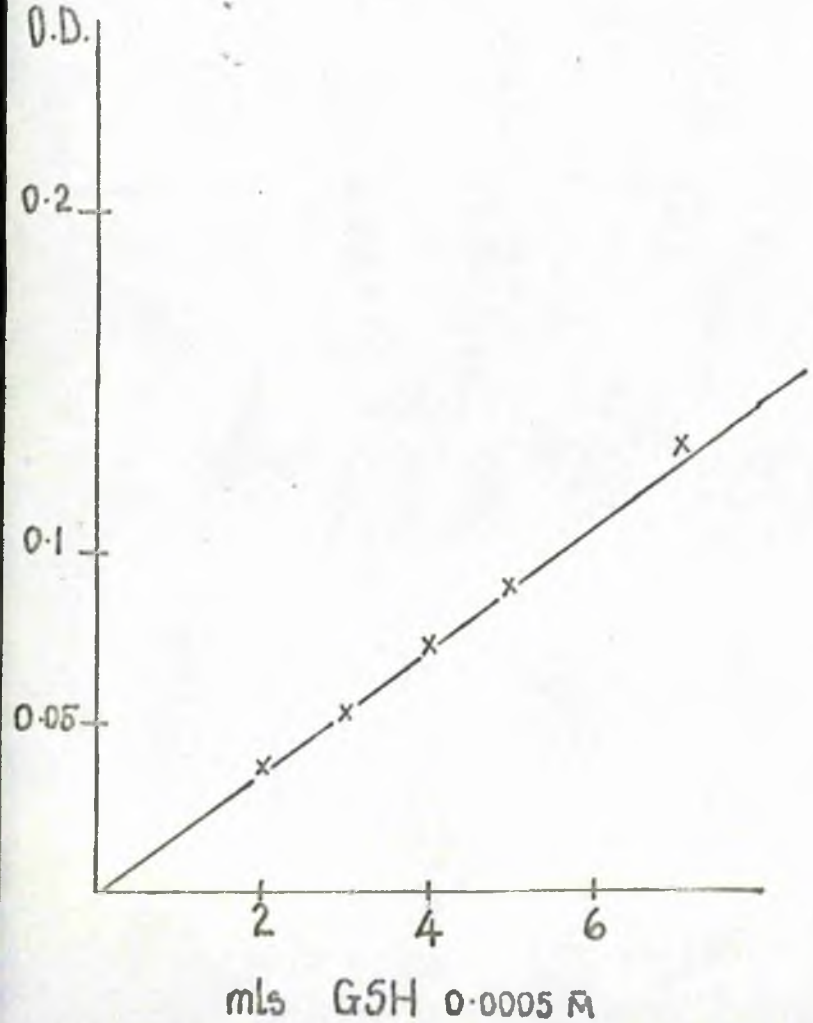
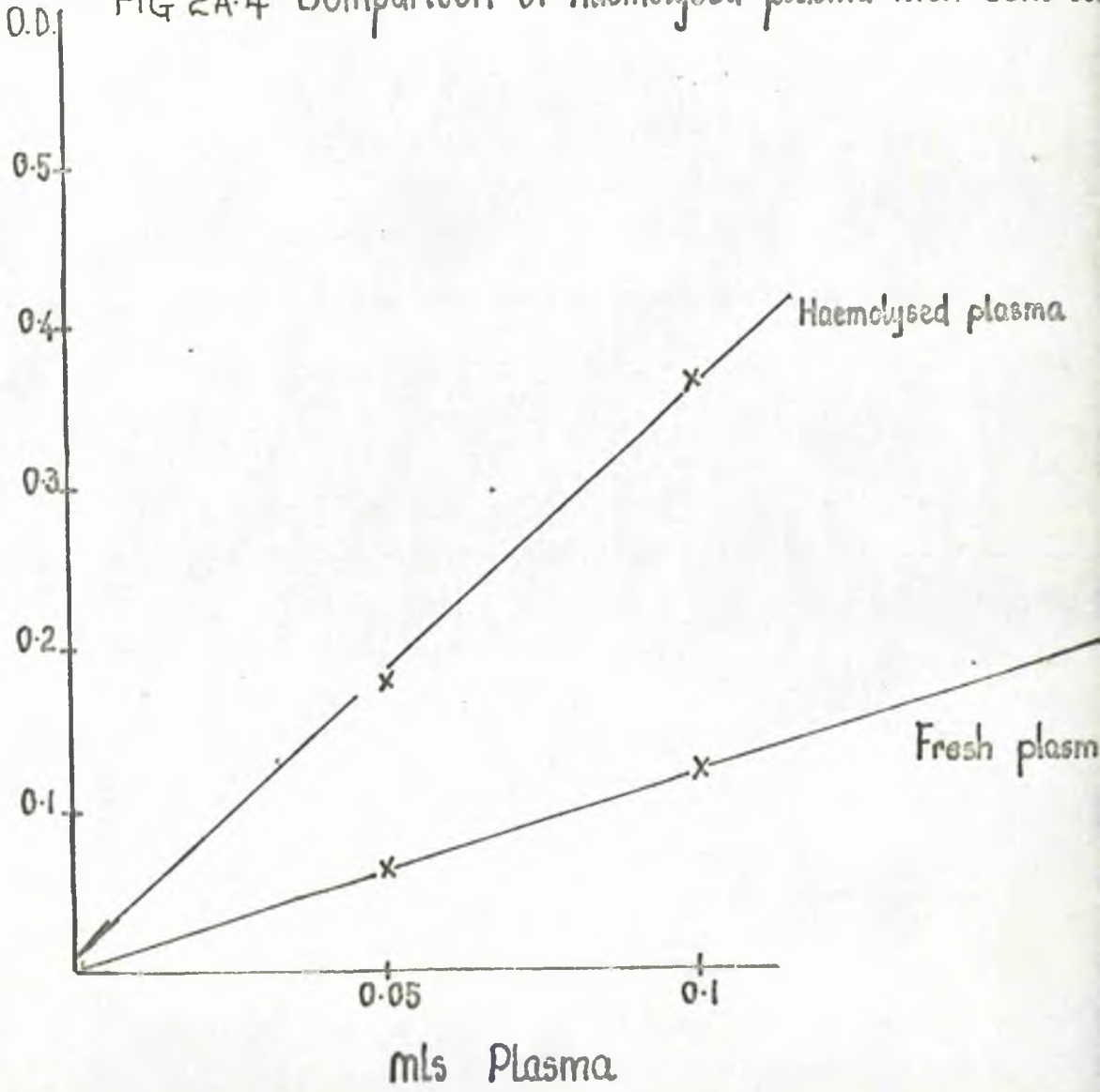


FIG 2A·4 Comparison of haemolysed plasma with control.



Comparison of fresh plasma with random haemolysed plasma Fig. 2A.4.

10-15 mls of fresh blood (heparinised) was divided into two aliquots. One was immediately centrifuged, the plasma withdrawn and a sulfhydryl analyses determined on 0.05 and 0.1 mls.

The other aliquot was shaken vigorously for 2½ hours on a shaker and the analyses of this performed likewise.

The results are seen in Fig. 2A.4 and the sulfhydryl value in the following table:

Table. Sulfhydryl concentration:

	Control Plasma.	Haemolysed Plasma
0.05 mls plasma	1.0×10^{-4} M	2.87×10^{-4} M
0.1 ml "	1.98×10^{-4} M	5.9×10^{-4} M

It is clearly seen that in this random haemolysed plasma the sulfhydryl concentration was raised some 300% compared with control. The significance of this will be elucidated in the discussion.

Optimal absorption of maleimide Fig. 2A.5

0.1 mls plasma was reacted as before with 9 mls NEM and 0.95 mls buffer, with the appropriate unreactive and blank tubes, and the absorption was read between 290 and 315 mμ using silica cells.

It is seen that at 300-305 mμ the test solution (reactant tube) is at its maximum and at 300 mμ the unreactive solution has its λ max at a discreet figure below its reactant

FIG. 2A.5 Optimal absorption of Maleimide.

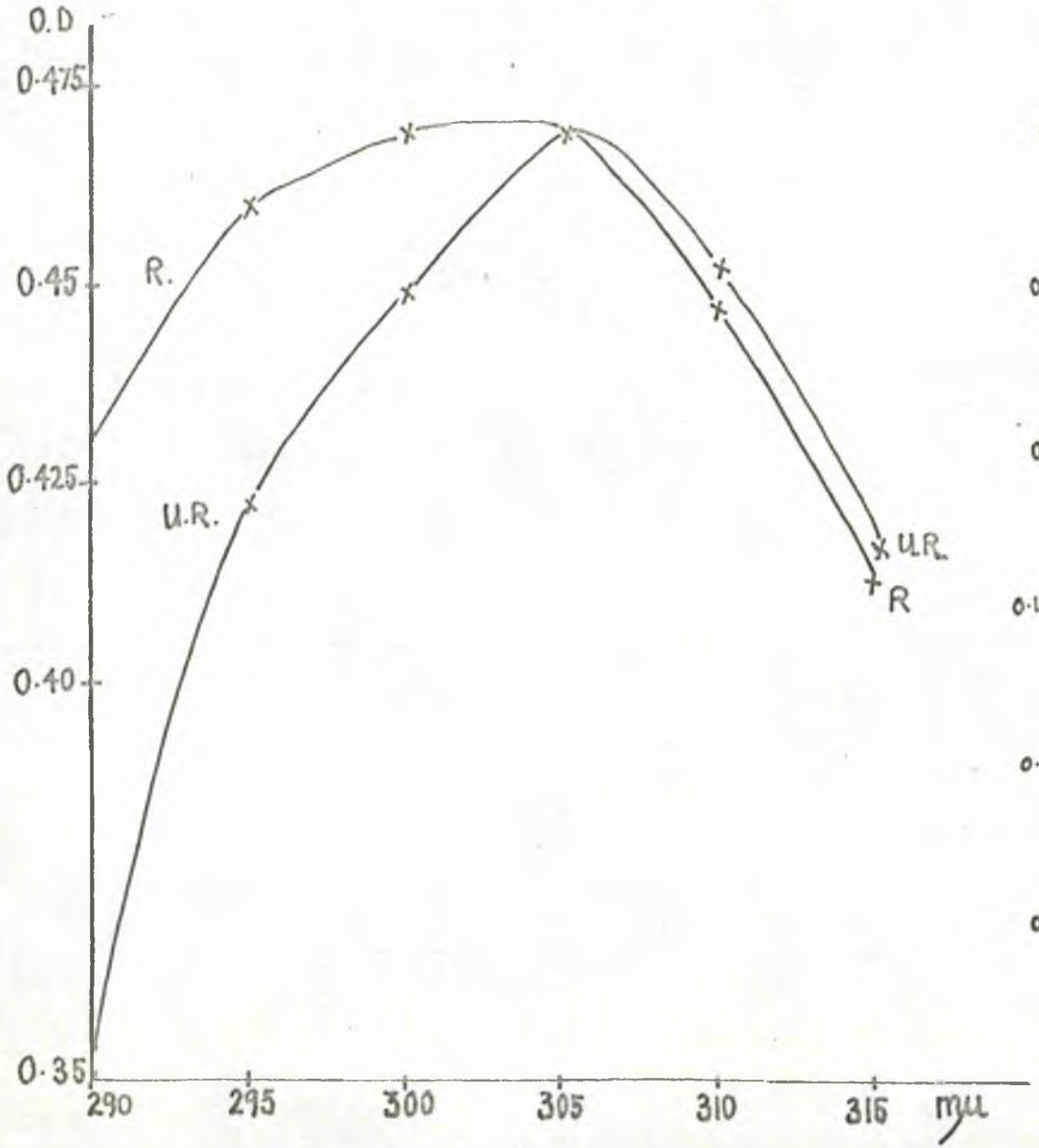
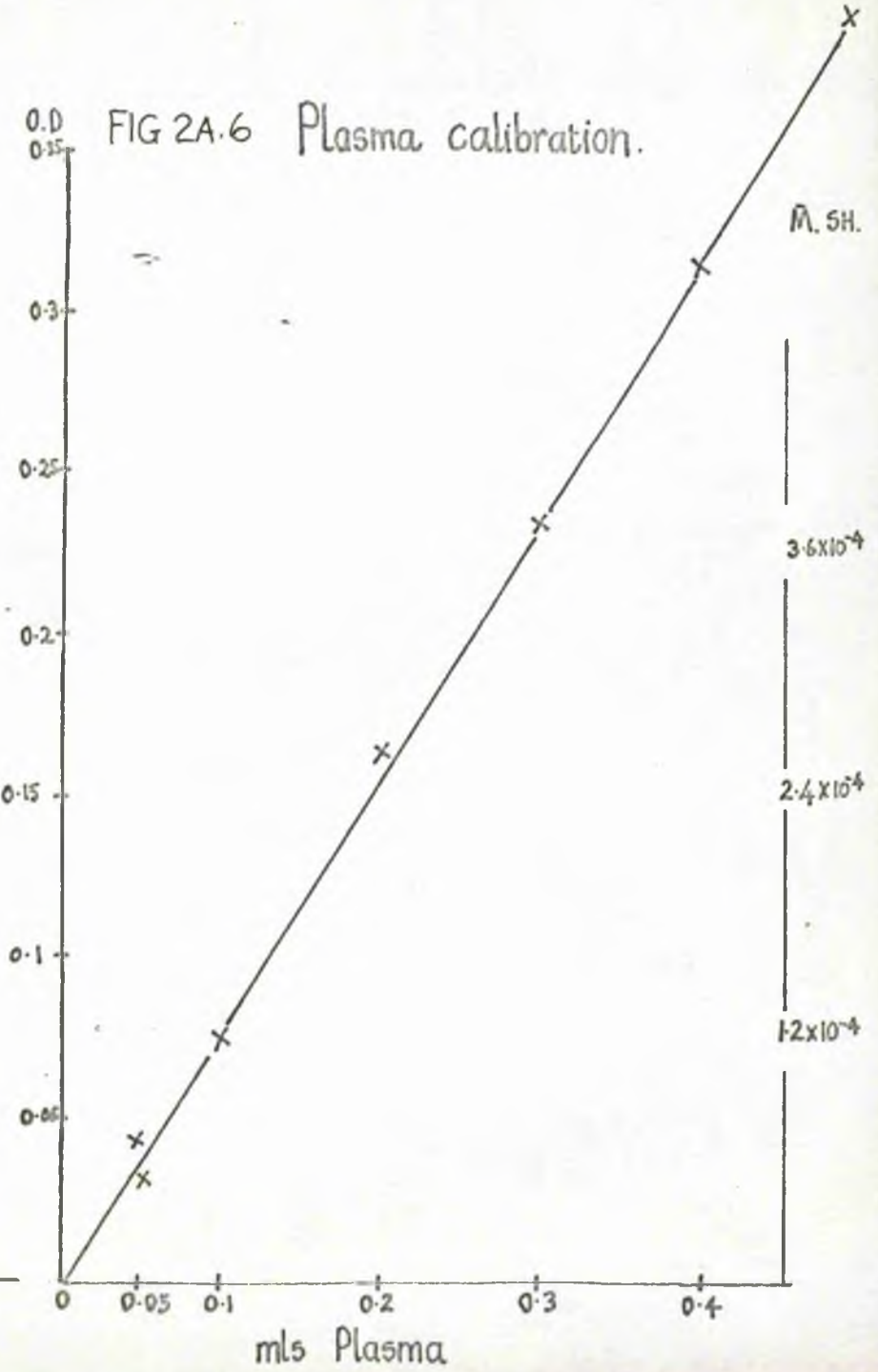


FIG 2A.6 Plasma calibration.



solution.

Below 300 mμ the absorption curves fall off rapidly and above 305 mμ the two curves virtually fuse and fall off rapidly as well.

The absorption of this maleimide work was therefore taken at 300 mμ, although the 300-305 mμ range may be used with equal success.

Varying plasma concentration with constant maleimide. Fig. 2A.6.

0.05-0.5 mls aliquots of plasma were reacted individually with maleimide (9 mls) and x mls of buffer to keep the total volume (10 mls) of reactants constant. As can be seen from Fig. 2A.6 there is excellent linearity.

The absorption readings are given in the table below. R = Reactant tube; U.R = Unreactive; and B = Blank.

Plasma	R	UR	R - UR =
0.05 mls	0.45	0.408	0.042
0.05	0.455	0.423	0.032
0.1	0.45	0.375	0.075
0.2	0.468	0.304	0.164
0.3	0.449	0.214	0.235
0.5	0.455	0.066	0.389

The 0.1 ml sample of plasma gives a sulfhydryl concentration of approximately 1.2×10^{-4} M, which is very consistent with other control values found elsewhere in this section.

The mean absorption of six readings is for 0.1 mls plasma using the above volume, 0.0789.

Plasma was reacted with maleimide in even greater dilution than 1:200 but very inconsistent results were obtained.

As is seen from fig. 2A.6 however Beers Law is obeyed excellently in plasma concentrations ranging from 1:200 (0.05 mls) up to 1:20 (0.5 mls).

Reaction of plasma with maleimide: Inverse concentrations: Fig. 2A.7

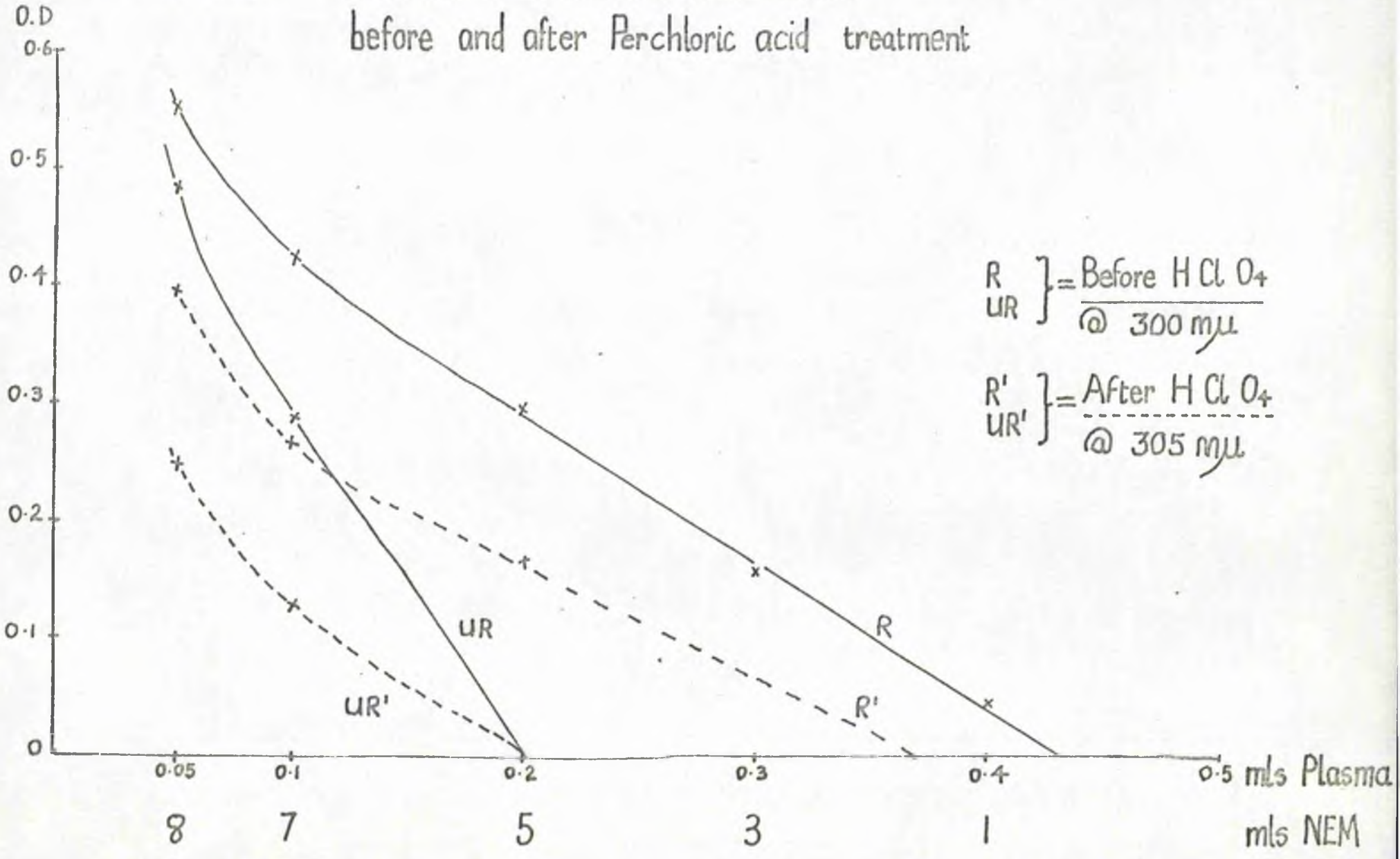
Increasing volumes of plasma were reacted with decreasing concentrations of maleimide. It is seen that a decrease in maleimide concentration leads to a decrease in optical density. In spite of this however initial linearity is seen in the results of 0.05, 0.1, and 0.2 mls plasma. See Fig. 2A.7. Total volume 10 mls as before.

Table 7

Plasma	(R)	(UR)	R-UR
0.05 mls	0.55	0.484	0.065
0.1	0.42	0.287	0.133
0.2	0.294	0.029	0.265
0.3	0.158	<0	-
0.5	0.045	<0	-

In the 0.3 and 0.5 ml plasma results, the maleimide concentration is too low and zero is reached in the unreactive solutions.

FIG. 2A. 7 Reaction of Maleimide with Plasma before and after Perchloric acid treatment



R, UR = Before $HClO_4$ @ 300 mμ

R', UR' = After $HClO_4$ @ 305 mμ

Precipitation of proteins after reaction of plasma with maleimide.

In Fig. 2A.7 as well can be seen, the same experiment but in this case the plasma proteins after reaction with maleimide were precipitated with perchloric acid ($HClO_4$) and the absorption of the filtrate read at 305 mμ. The corresponding R and UR values are seen as R' and UR'.

This method was not pursued any further as very accurate and reliable results had been obtained in the foregoing procedures It will be seen however that the Reactive and unreacted lines are virtually parallel rather than divergent indicating poor linearity with increase in plasma concentration. This is borne out in the R - UR figures.

Table 2A.7

Plasma	R	UR	R-UR
0.05	0.389	0.243	0.146
0.1	0.265	0.126	0.139
0.2	0.165	0.0025	0.1625
0.3	0.0575	-0	?
0.4	-0	-0	?

However if difficulty was experienced with absorption of a reacted protein with maleimide, then a calibration curve of this perchloric acid precipitation method would be of first priority.

The effect of prolonged oxygenation on blood fig. 2A.8

30 mls of fresh blood and 4 mls saline (0.9%) containing a few drops of silicone fluid was actively oxygenated for 6-7 hours and small aliquots e.g. 0.02-0.1 mls taken off at hourly intervals and analysed immediately. Saline - approximately 12% was added to the blood initially to mimic the degree of dilution seen in extra corporeal perfusion.

The analysis of the experiment was performed with not only maleimide but also DTNB as sulfhydryl reagents. The latter result is seen in the DTNB section but the conclusions are not complimentary.

The oxygen rate was 4-5 litres/min.

It is seen from fig. 2A.8 that there is a slow but definite fall in detectable sulfhydryl groups when oxygen is actively bubbled through blood for several hours. The importance of this in perfusion work is of course centred around changes occurring in the first 4 hours, - few perfusions are longer than this.

The effect of bubbling nitrogen through whole blood gives rise to an increase in sulfhydryl groups, the curve of which is roughly sigmoid, fig. 2A.8.

The individual results of both curves are the mean values of three or four readings.

FIG. 2A.8 The effect of prolonged Oxygen and Nitrogen treatment on reactive sulfhydryl groups @ 300 mμ

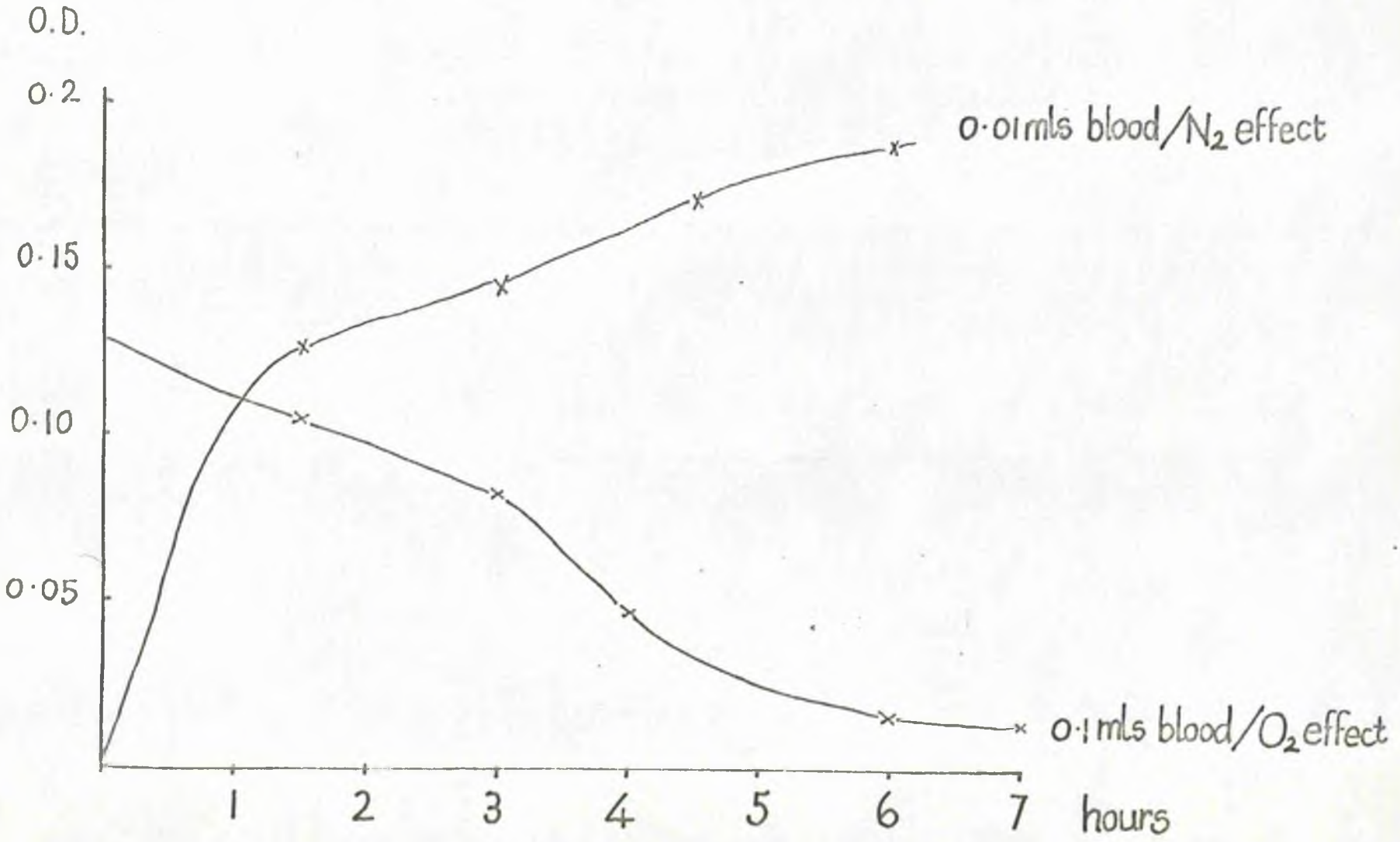
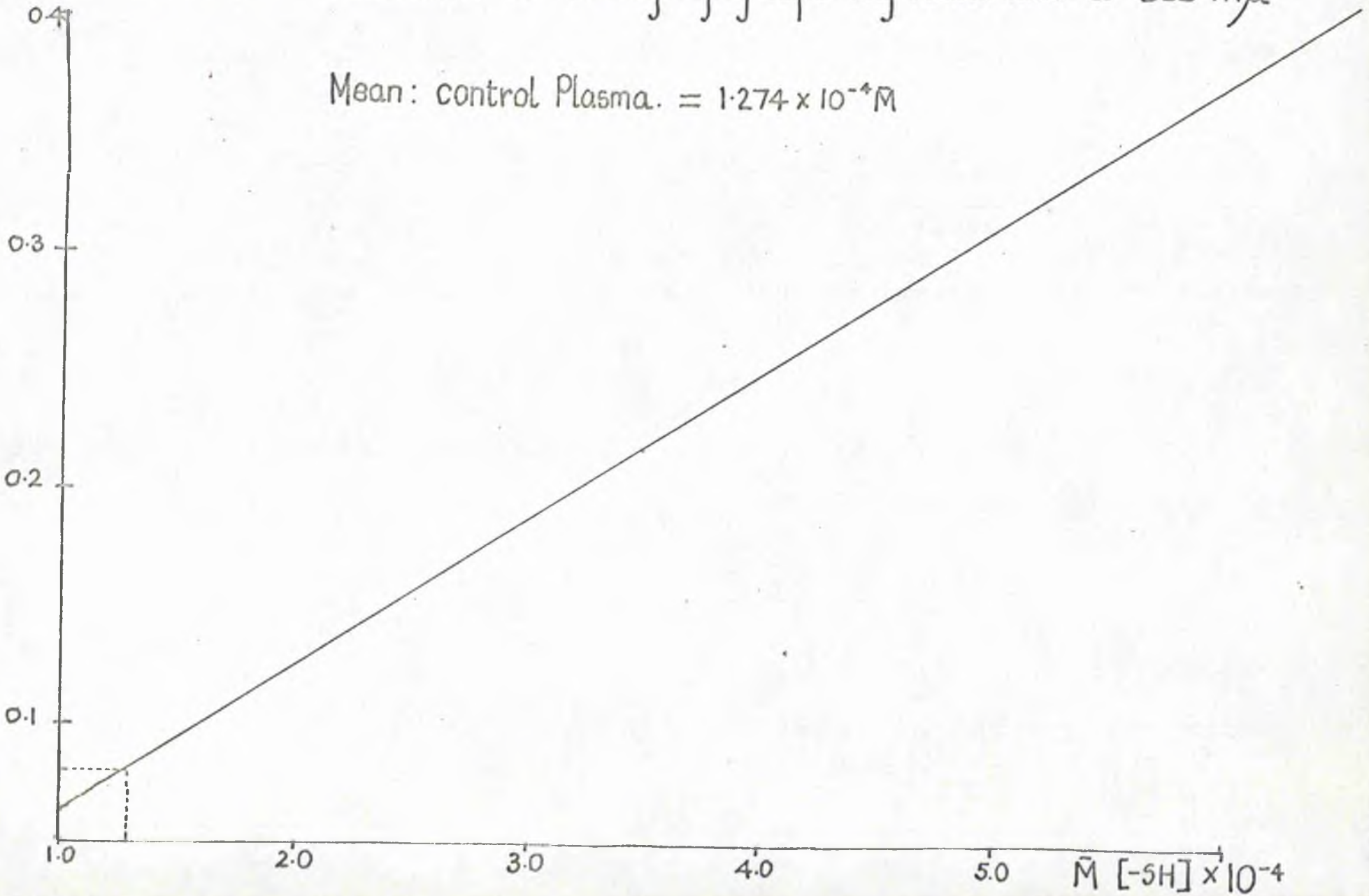


FIG. 2A.9 Calibration of sulfhydryl groups by Maleimide @ 300 mμ

Mean: control Plasma. = 1.274×10^{-4} M



Sulfhydryl concentration of haemolysed blood. Fig. 2A.9

This experiment was an attempt to find a value for complete denaturation by physiological means rather than by using denaturing agents which add complications such as reaction with sulfhydryl reagents, dilution and λ max changes.

20 mls. of blood was diluted to 25 mls with water (dist.), placed on a shaker, and shaken vigorously for 3 hours at room temperature. The sample was then centrifuged and the plasma removed.

Another sample of the blood diluted 20% with isotonic saline was used as control. This sample was estimated for sulfhydryl concentration immediately and not shaken. After correction for dilution the grossly haemolysed plasma (0.05 mls) gave a value of 6.75×10^{-1} mM/SH/L. This is equivalent to an O.D. reading of 0.419 (R-U.R.).

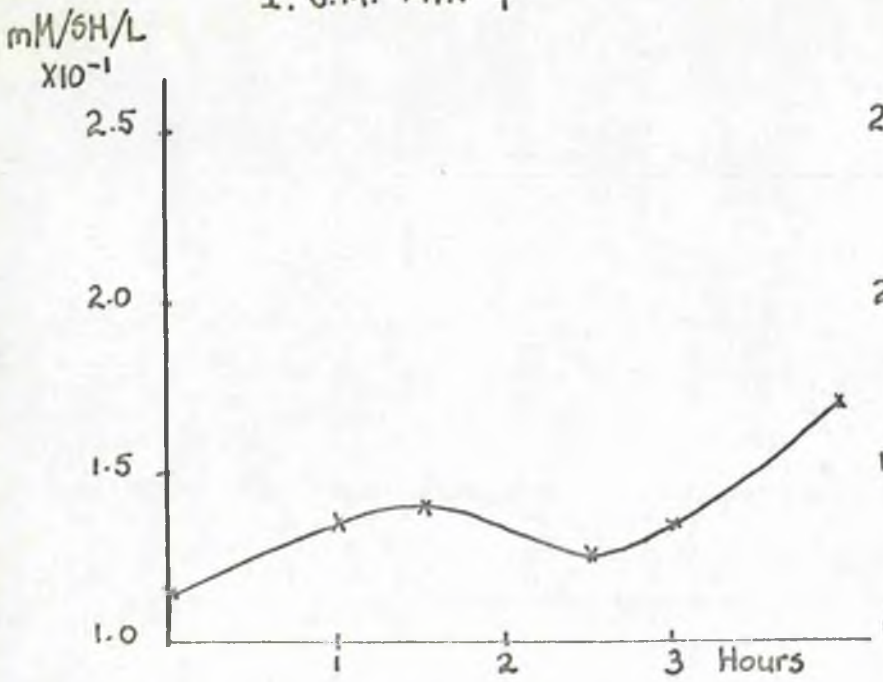
If this figure is taken as a theoretical approximately 100% haemolysis, then it is possible for a calibration graph to be drawn up relating - SH content and % haemolysis of plasma, Fig. 2A.9.

Plasma - SH changes in operations.

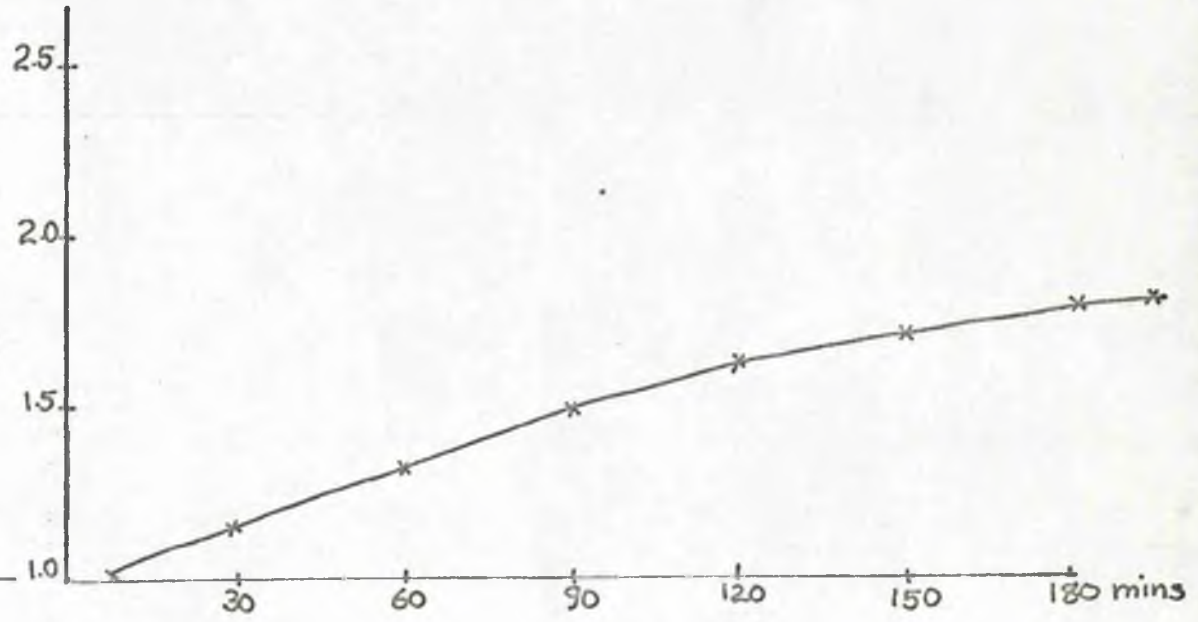
It will be seen from the results of plasma samples from perfusions, general surgery and the Melrose experiment (figs. 2A.10-2A.14), that the - SH groups increase in concentration, whic is largely time dependent. The type of increase - e.g. linear, parabolic, sigmoidal etc. - is not constant due to inevitable small changes in blood volume and constituents. It is reasonable

FIG. 2 A·10 NEM: Plasma -SH (0·05 mls)

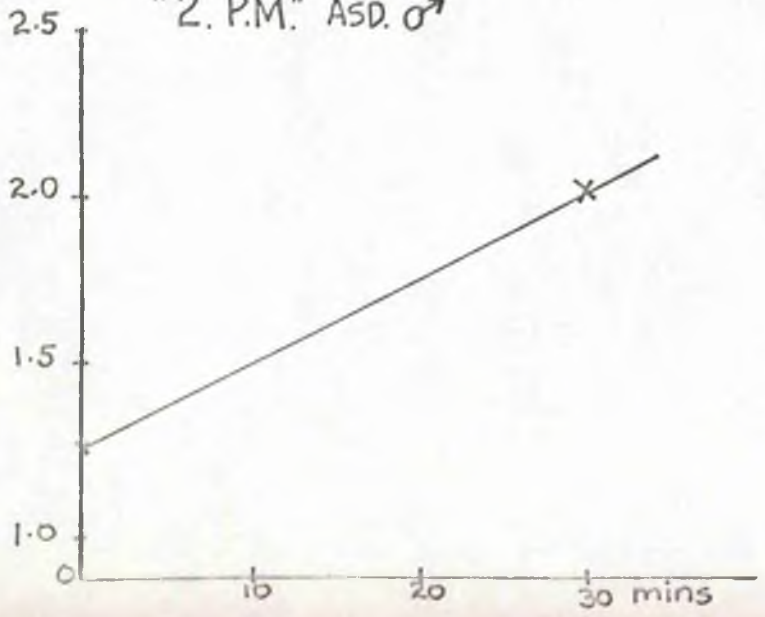
"I. J.M." MVR ♀



"4. "M.Ch" AVR ♀



"2. P.M." ASD. ♂



"3 B.K." ASD/VSD ♂

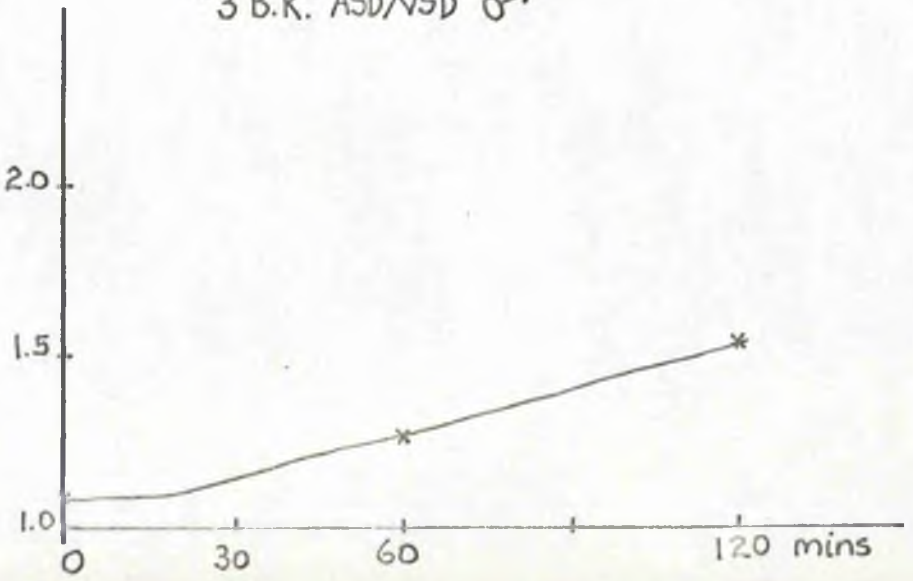
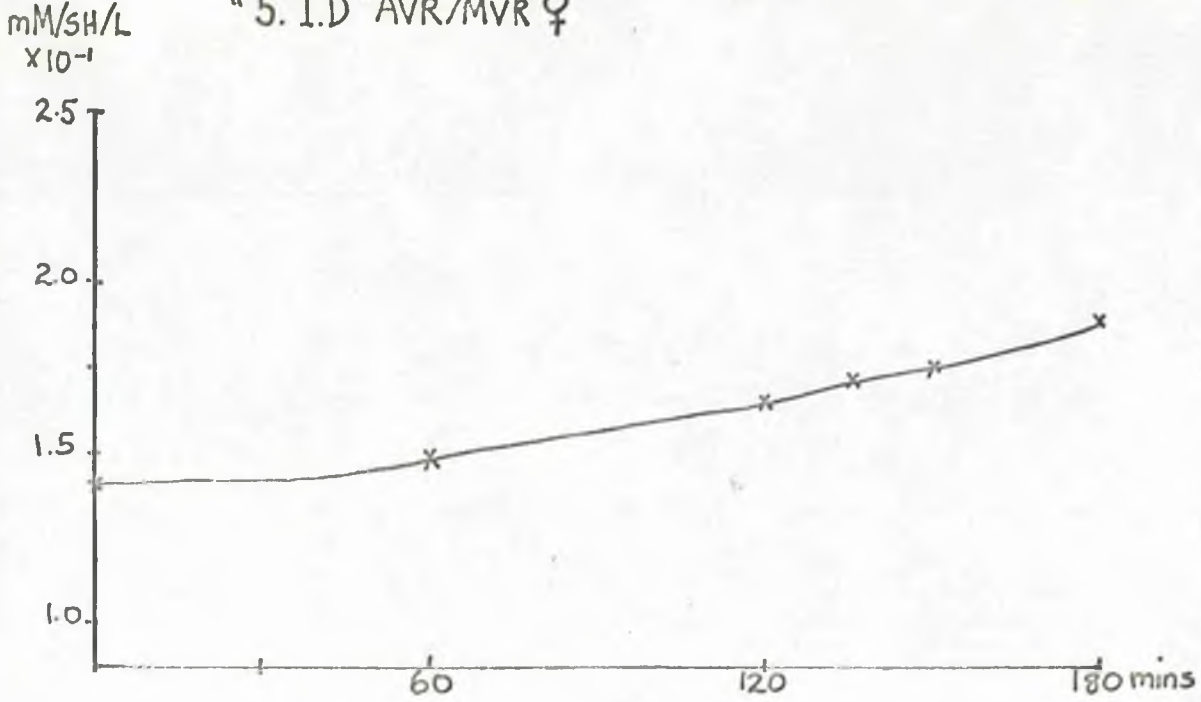


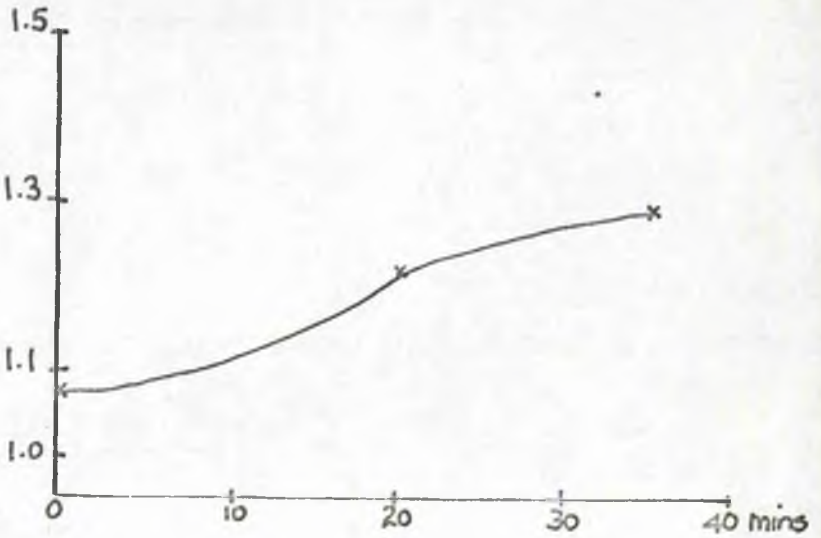
FIG. 2 A·II

NEM: Plasma -SH. (0·05 m/s)

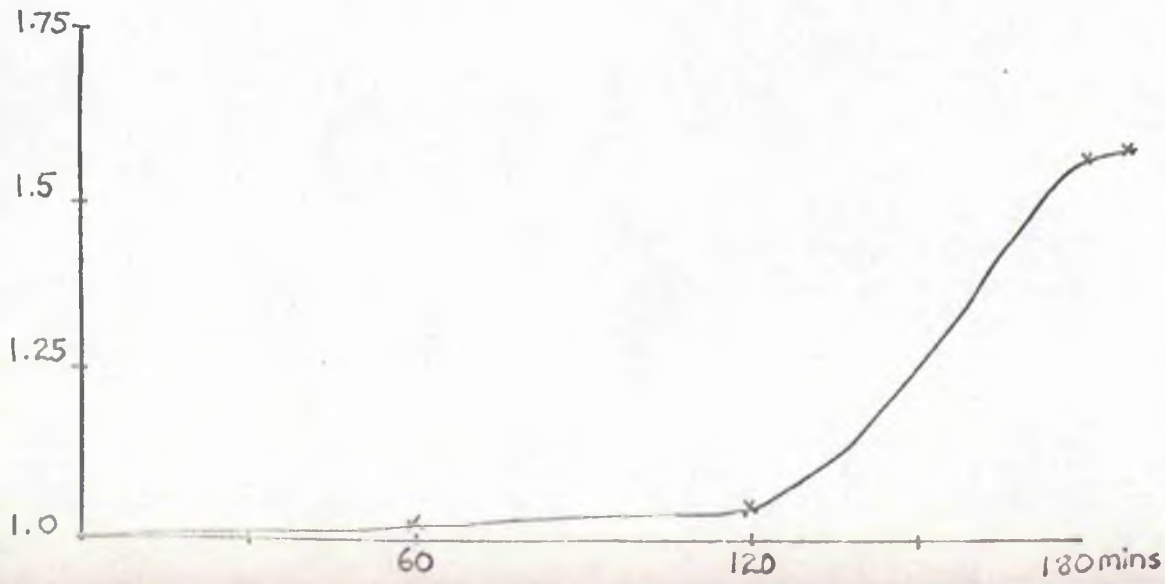
"5. I.D" AVR/MVR ♀



"8. F.K" M.VR ♂



"6. S.S." AVR ♀



"7. D.M" AVR ♂

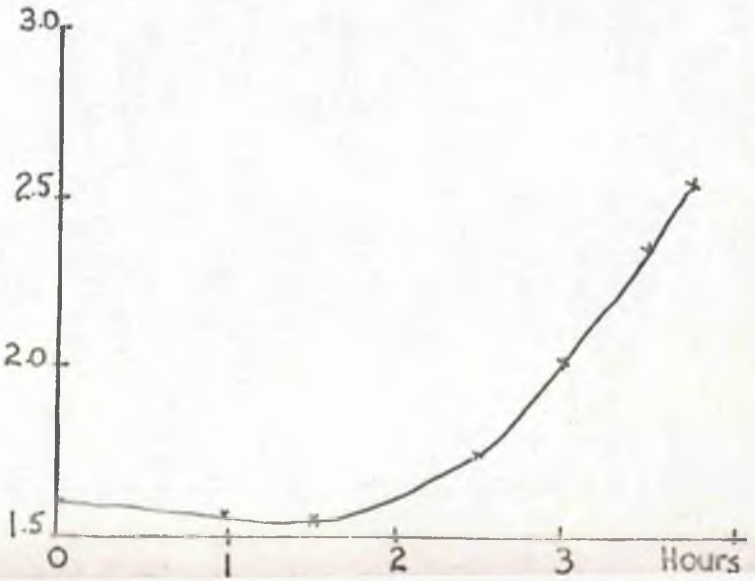
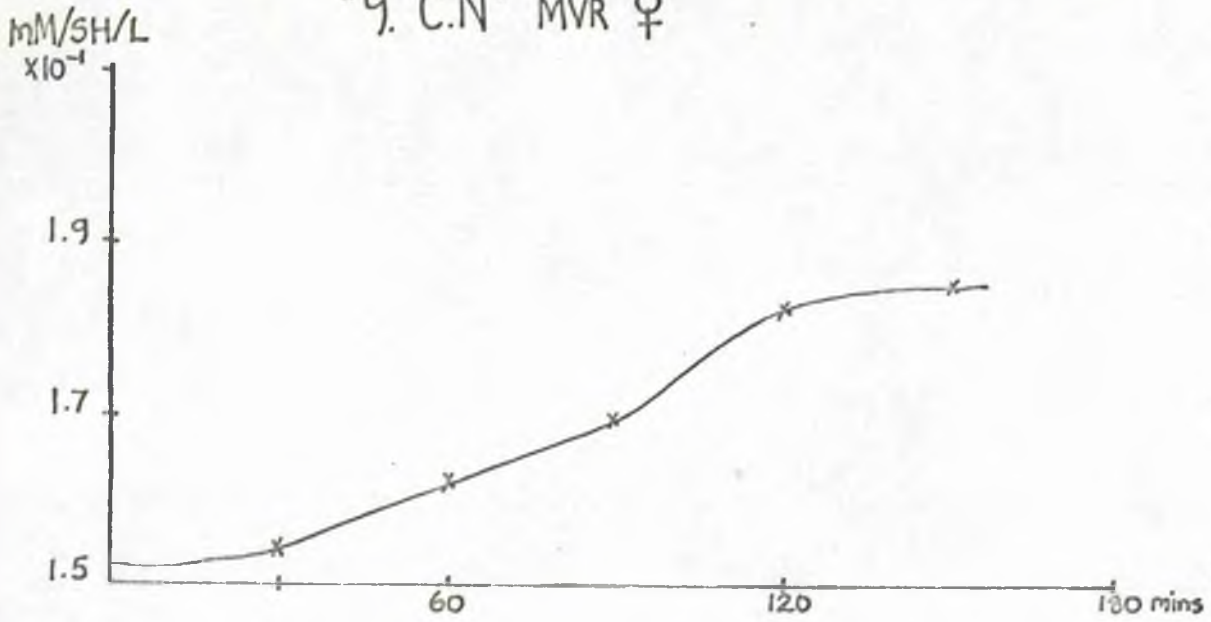
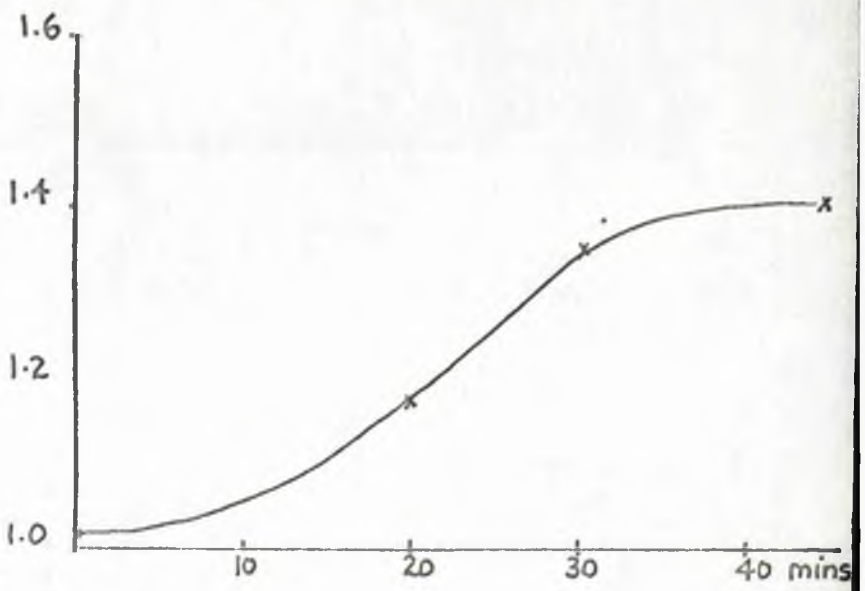


FIG. 2 A·12 NEM: Plasma -SH (0·05 mls)

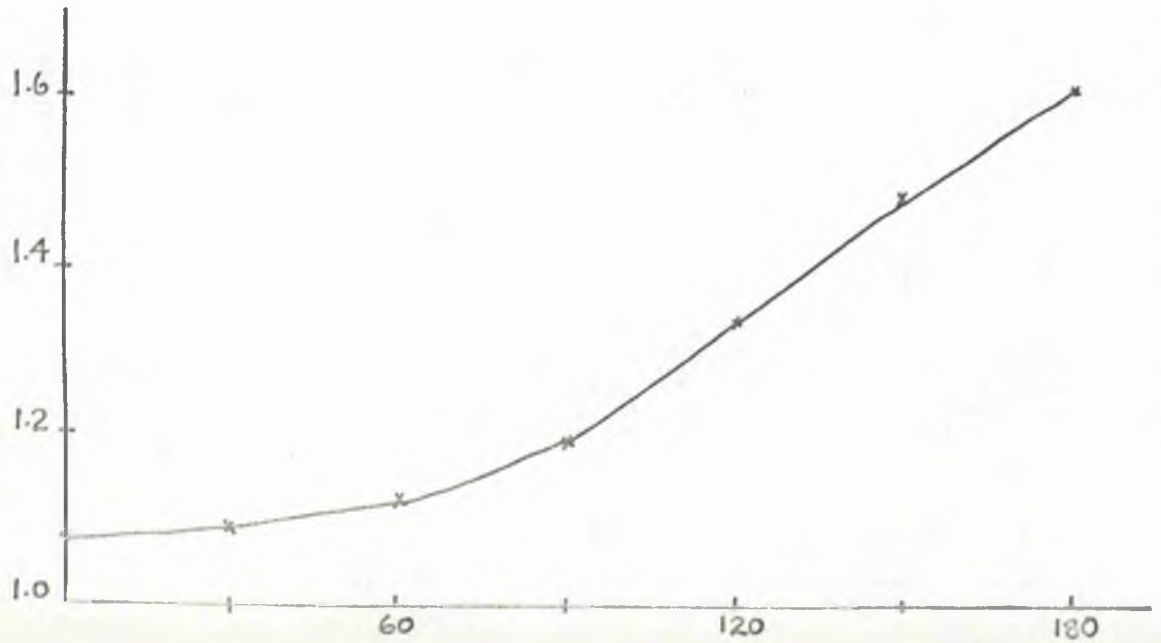
"9. C.N" MVR ♀



"12. D.S" ASD ♂



"10. R.M" ♂ MVR



"11. A.C." MVR ♀

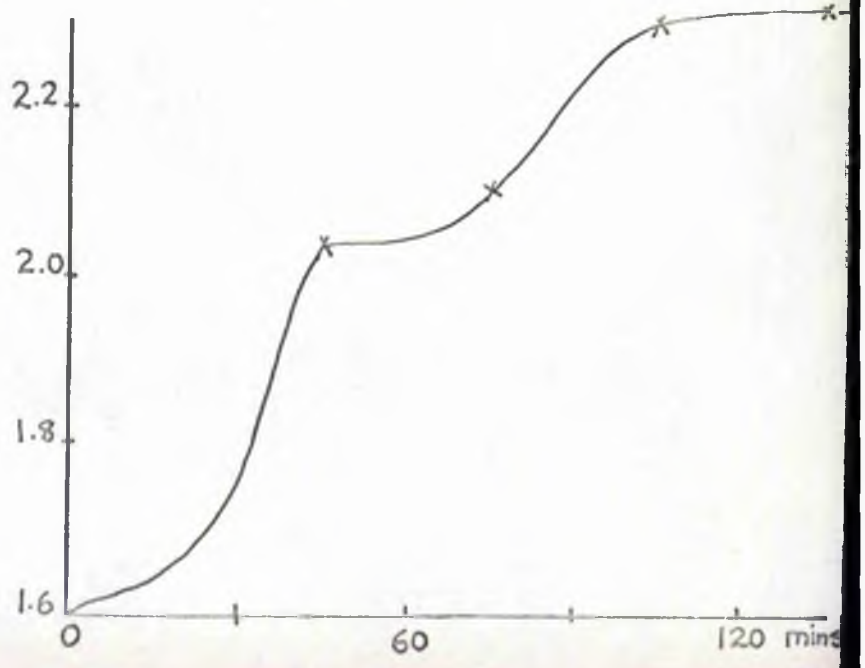


FIG. 2 A·13 NEM: Plasma -SH. (0·05 mls)

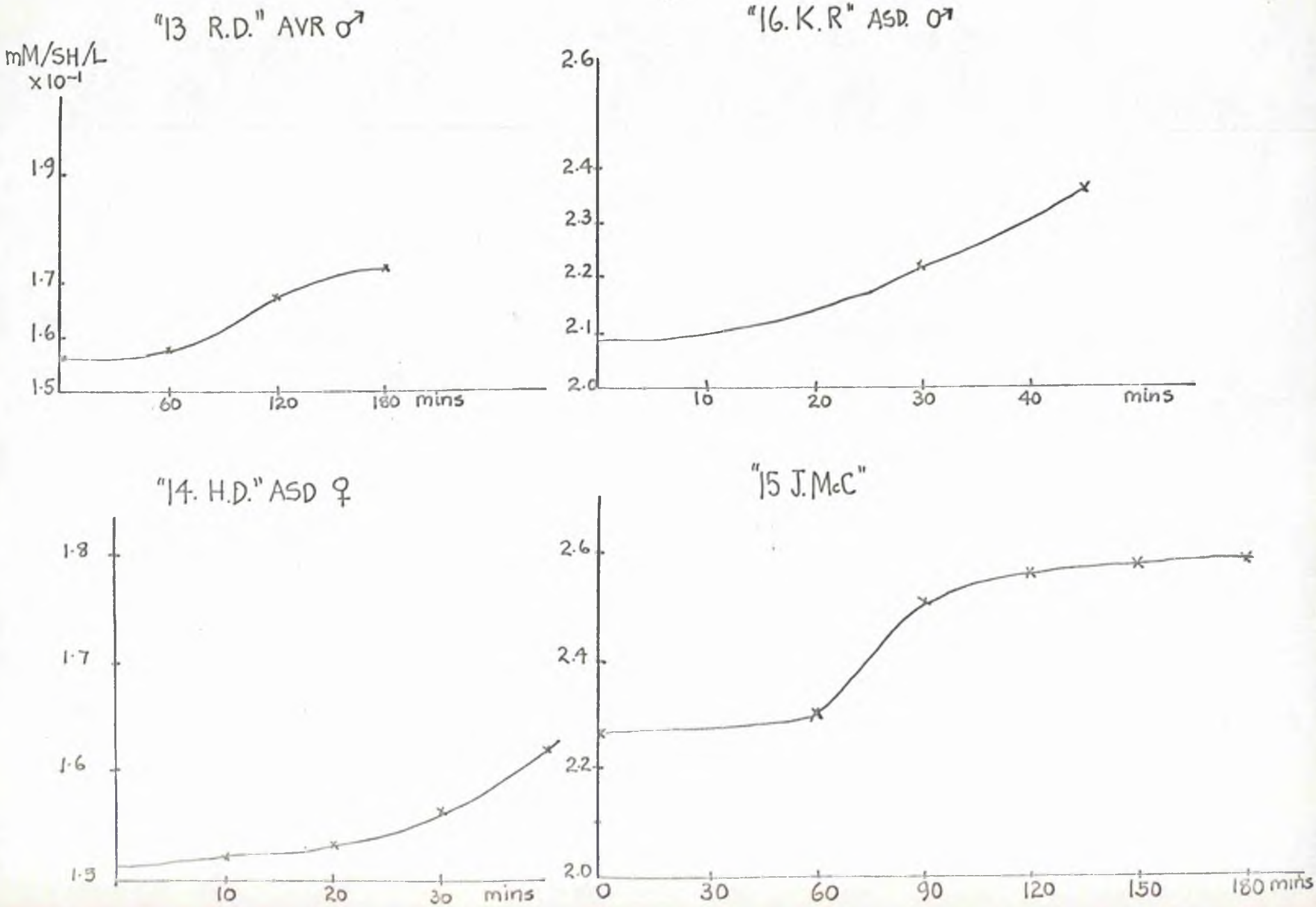
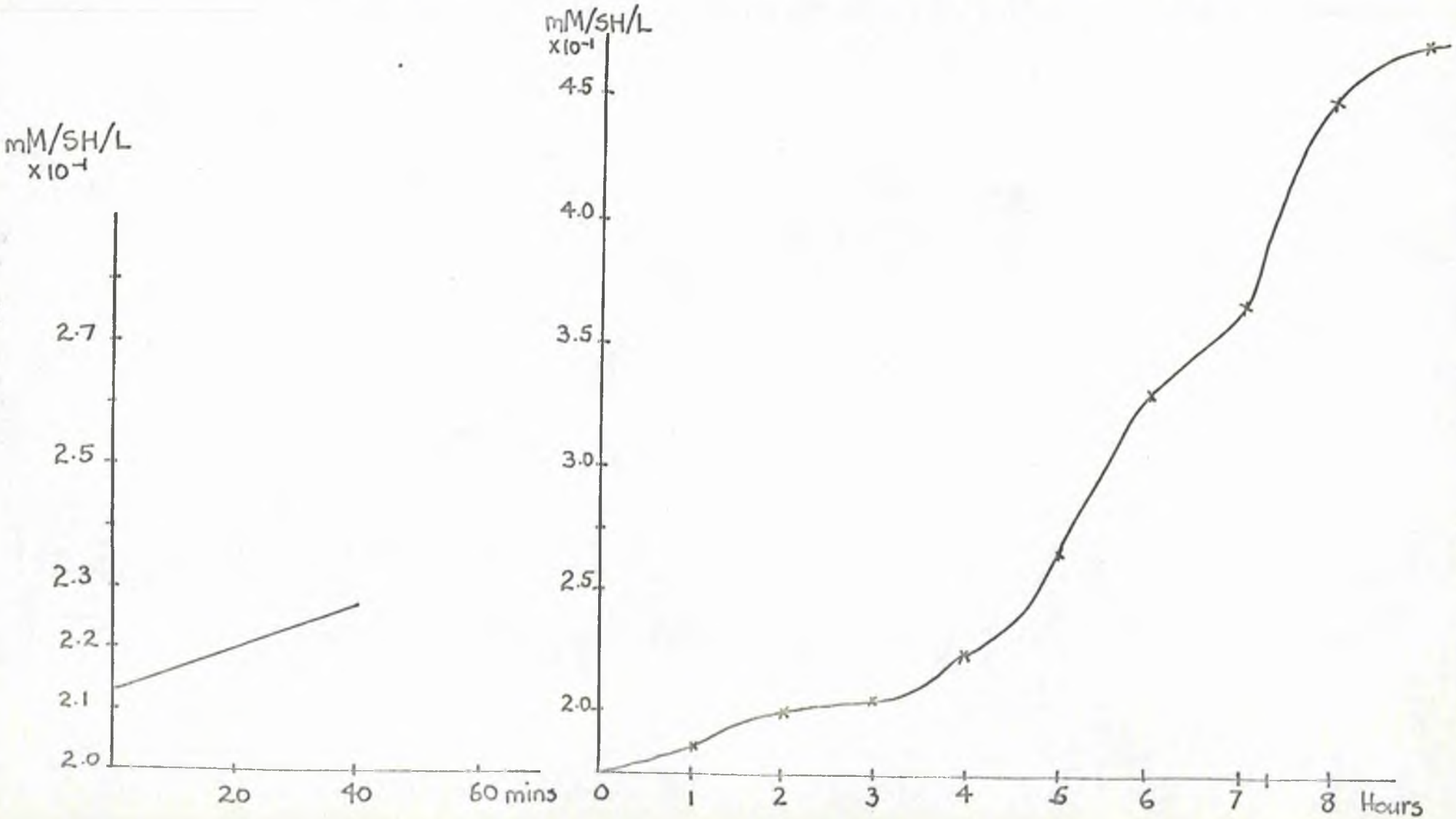


FIG. 2A·14 NEM: Plasma -SH (0·05mls)

"17. D.M." Ant. Resection. ♂

Melrose Experiment : Control.



to assume that the parabolic curve probably represents the 'true change' since it is seen in the Melrose experiment.

The individual - SH values from each patient may be seen in Appendix 2.

DISCUSSION

The specificity of NEM in the detection of - SH groups is well shown in the section providing that the concentration of NEM is sufficiently high - above 30% - using the concentrations mentioned. Assays were done on 0.1 mls plasma using a total volume (NEM, buffer, plasma) of 10 mls.

It is quite clear from the results that haemolysis leads to a vast increase in - SH groups and that they are linearly related.

The effect of oxygen and nitrogen bubbling upon blood is complex, for proceeding side by side is the increase of sulfhydryl groups by denaturation, haemolysis and the formation of sulfhydryl groups de novo from easily hydrolysable disulphide bonds or some other unstable linkages(s) which can give rise to sulfhydryl groups under relatively mild conditions.

Against these factors are the oxidation of sulfhydryl to disulphide and beyond, the loss of hydrogen sulphide by β elimination and ? reversibility of denaturation.

It would appear that in the oxygenation graph - the detectable reactive sulfhydryl groups that are being made available are being destroyed at a rate in excess of their formation, by chiefly oxidation. It must be borne in mind that

only a relatively small volume of blood was being used (30 mls) - in comparison with perfusate volume - and this may well be an important factor because in the latter volume there is greater buffering ability of the blood.

The nitrogen graph on the other hand would indicate either

1) that no detectable destruction or alteration of sulfhydryl groups e.g. oxidation, is taking place or
2) that any loss of sulfhydryl groups occurring in this experiment is proceeding at a rate very much less than the rate of formation or availability of these groups.

The Melrose experiment shows the dramatic increase in plasma - SH content while the blood was being continuously circulated round the closed circuit for up to 9 hours. The initial - SH concentration was 1.87×10^{-1} mM/SH/L. This rose slowly over the first 3-4 hours, then a much more rapid increase - which was roughly linear - took place, giving a final value after 9 hours of 4.72×10^{-1} mM/SH/L. This latter value is equivalent to - SH values seen in plasma with over 50% haemolysis.

The increase in plasma haemolysis may be seen in Figs. 2A.14 and 5.5.

Operation results. Although all the operations showed a definite increase in - SH content, it was very much less than that seen in the Melrose experiment, although the first 3 hours of the latter mimic the operation changes very closely.

The increase in - SH content of plasma from these operat-

ions is equivalent to - SH values seen in plasma with 5-15% haemolysis.

It will be seen that the increase in plasma - SH content in the Melrose experiment mimics the degree of haemolysis seen in the photograph of the serial plasmas Fig. 5.5. The estimation of haemolysis by the Cyanomethaemoglobin method was not done on these specimens.

It is thus reasonable to assume that the increase in - SH content of blood in operations - and especially perfusions - is largely due to the degree of haemolysis present. Lesser contributing factors are probably protein denaturation and some degree of contribution from the metabolism of sulphur amino acids. The extent and importance of these factors will be discussed in the final discussion.

The control - general surgery operations gave similar results to the perfusions. The - SH values are at a slightly higher level due probably to less initial haemodilution being required.

CONCLUSION.

1) N-Ethyl maleimide is well suited for routine, serial estimation of plasma - SH groups providing it is in sufficient concentration - above 30% (1×10^{-3} M) in the total reactant mixture.
2) There is a linear relationship between plasma - SH and Haemoglobinaemia.
3) Using only small volumes of blood the results of gassing

(O_2+N_2) appeared rather equivocal.

4) The Melrose experiment gave an increase in plasma - SH content which is equivalent to over 50% haemolysis over the 9 hour period.

5) The operation results gave plasma - SH values equivalent to 5 - 15% haemolysis over the 3 hour operation period. These results were similar to the first 3 hours of the Melrose experiment.

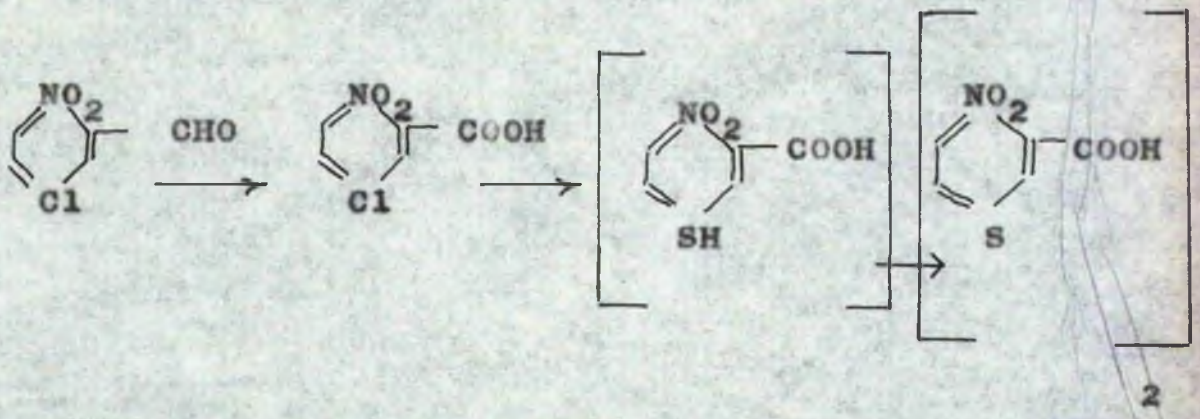
Section 2B: DTNB

INTRODUCTION

5.5' Dithiobis - 2 nitrobenzoic acid

$[-S.C_6H_3(NO_2)COOH-]_2$ = 396.35

DTNB is another specific reagent for sulfhydryl assay which may be synthesised from 2 - nitro 5 chloro benzaldehyde.



It is the water soluble derivative of bis p. nitro phenyl disulphide and because of this property it was used in preference to the latter in the initial preparatory work on the selection of the best sulfhydryl reagent for - SH assay of blood.

DTNB is a very toxic compound and care must be exercised in weighing and related techniques. It remains stable for many weeks in phosphate buffer and thus does not require constant standardisation.

Its use in the assay of serial samples is accurate and well suited, for after an hours reaction between the blood and DTNB, the results of as many samples as is needed may quickly be read.

The Molar Extinction coefficient is 13,600/M/cm.

Method. A 0.01 M standard DTNB solution was made up in 0.1 M phosphate buffer, pH 8.0

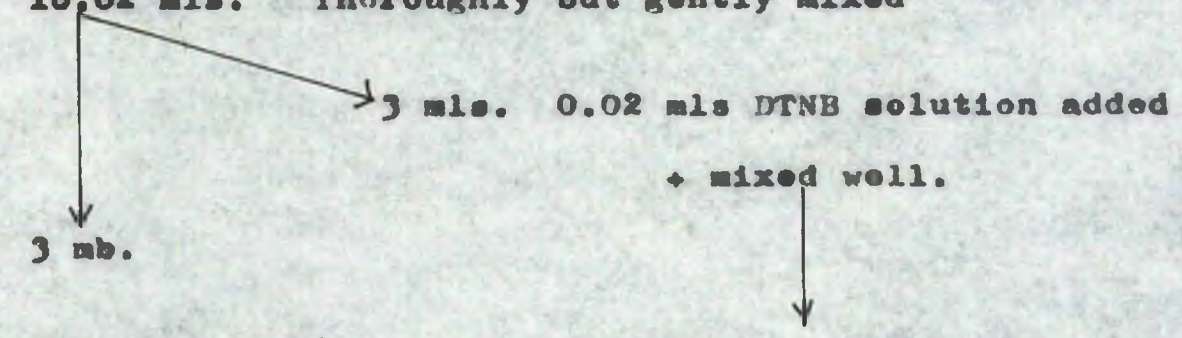
(39.6 mgms DTNB/10 mls phosphate buffer).

0.02 mls fresh whole blood were added to

9 mls water and

1 ml phosphate buffer

10.02 mls. Thoroughly but gently mixed



$$C_o = \frac{A}{\epsilon} D = 36.8\ A$$

where C_o = original concentration.

A = Absorption at 412 mμ.

ϵ = Extinction coefficient 13,600/M/cm.

D = Dilution factor.

0.02 mls whole blood was used for most experiments. Deviations from this volume were made however and the appropriate dilution factor used.

For fresh normal whole blood the - SH content by this method is

$$C_o = \frac{0.1275}{13.6} \times \left[\frac{10.02}{0.02} \times \frac{3.02}{3} \right] \text{ mm/SH/L.}$$

- SH estimations of urine.

$$= \frac{0.1275}{13.6} \times 504.3 = 0.1275 \times 36.8$$

$$= 4.75 \text{ mM/SH/L}$$

Dilution factor table.

Vol. blood mls.	Volume DTNB mls.	Dilution Factor
0.02	0.02	36.8 x A
0.02	0.04	37.21 A
0.04	0.02	18.58 A
0.04	0.04	18.64 A

Ellman, 1959 suggests a simple method for - SH detection in urine. Fresh urine is adjusted to pH 8.0, Centrifuged, 3 mls of the supernatant taken and put into each of 2 cells. One is used as a blank and 0.02 mls 0.01 $\bar{M}$ DTNB solution, pH 8.0 is added to the other cell. A random urine gave a result of 0.038 in M/SH/L.

RESULTS.

Sulfhydryl standardisation using Glutathione (red)

Three standard glutathione solutions were made each containing 5, 7.5 and 10 mgms/ml. This is equivalent to 100, 150 and 200 µg/0.02 ml.

0.02 ml aliquots of these standards were reacted with DTNB as above and absorption of the reaction taken at 10 minute intervals for 1 hr. With these standards equilibrium was obtained almost immediately and the colour stable for at least an hour.

FIG. 2B.1 Determination of optimal absorption using Glutathione.

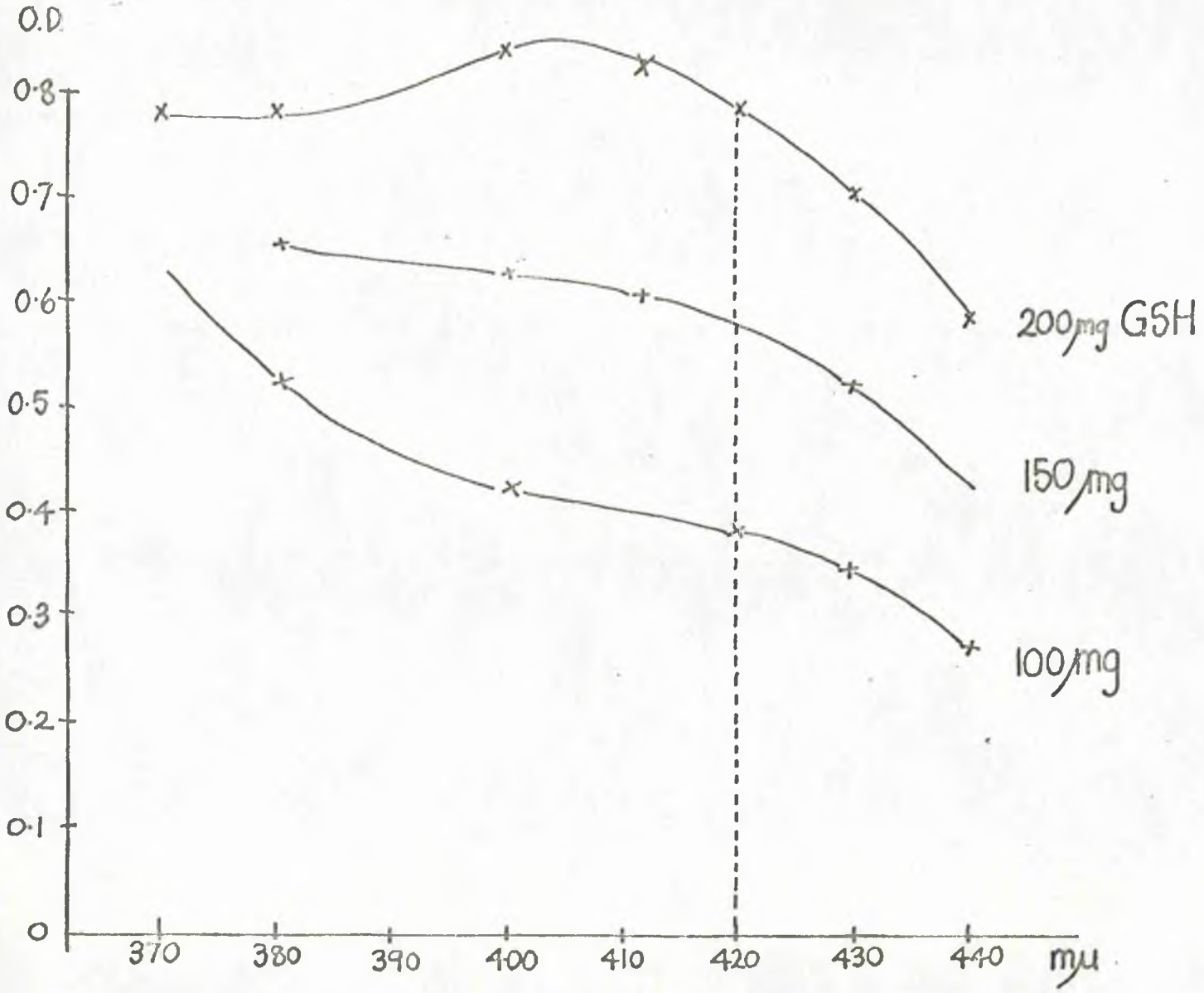
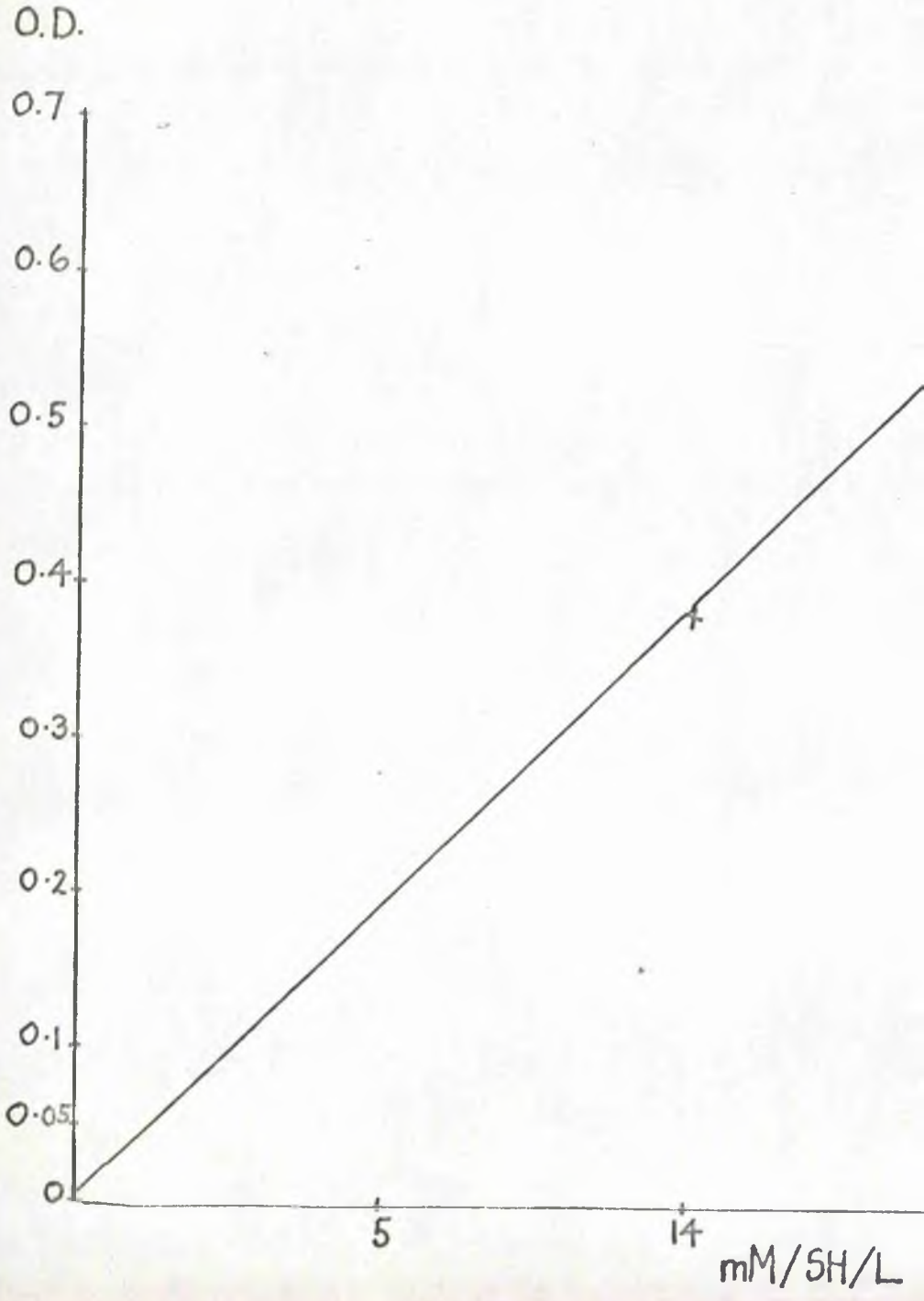


FIG. 2B.2 Calibration of Glutathione




21
28

→	100 µg	150 µg	200 µg	● 420 mµ
10	.384	.573	.773	
20	.381	.575	.773	
30	.38	.576	.771	
40	.38	.575	.770	
50	.38	.575	.77	
60	.379	.574	.77	

This experiment shows that approximately 33 µgms of glutathione is equivalent to the number of reactive sulfhydryl groups available in whole blood (0.02 mls).

Absorption at 420 mµ of 100 µgm GSH/0.02 ml = 0.38

150 " " = 0.574

200 " " = 0.77

Therefore = 13.98, 21.12 and 28.3 mM/SH/L respectively See Fig. 2B.1.

The absorption graph of GSH and DTNB is seen in fig. 2B.2 and it is clear that Beers Law is only true between 408—425 mµ.

Sulfhydryl determination of fresh blood and stability with time

The reaction of whole blood with DTNB is similar to the glutathione calibration above except that stable absorption is reached only after 30 minutes and lasts for approximately another 30 minutes, 2B·3.

0.02 mls samples of blood gave a concentration of 4.6 mM/SH/L which is slightly less than that reported by Ellman et al., 1958 (mean 6.05 mM/SH/L).

FIG 2B.3 The stability of Reactive Sulfhydryl groups of Blood (0·02 mls) with time.

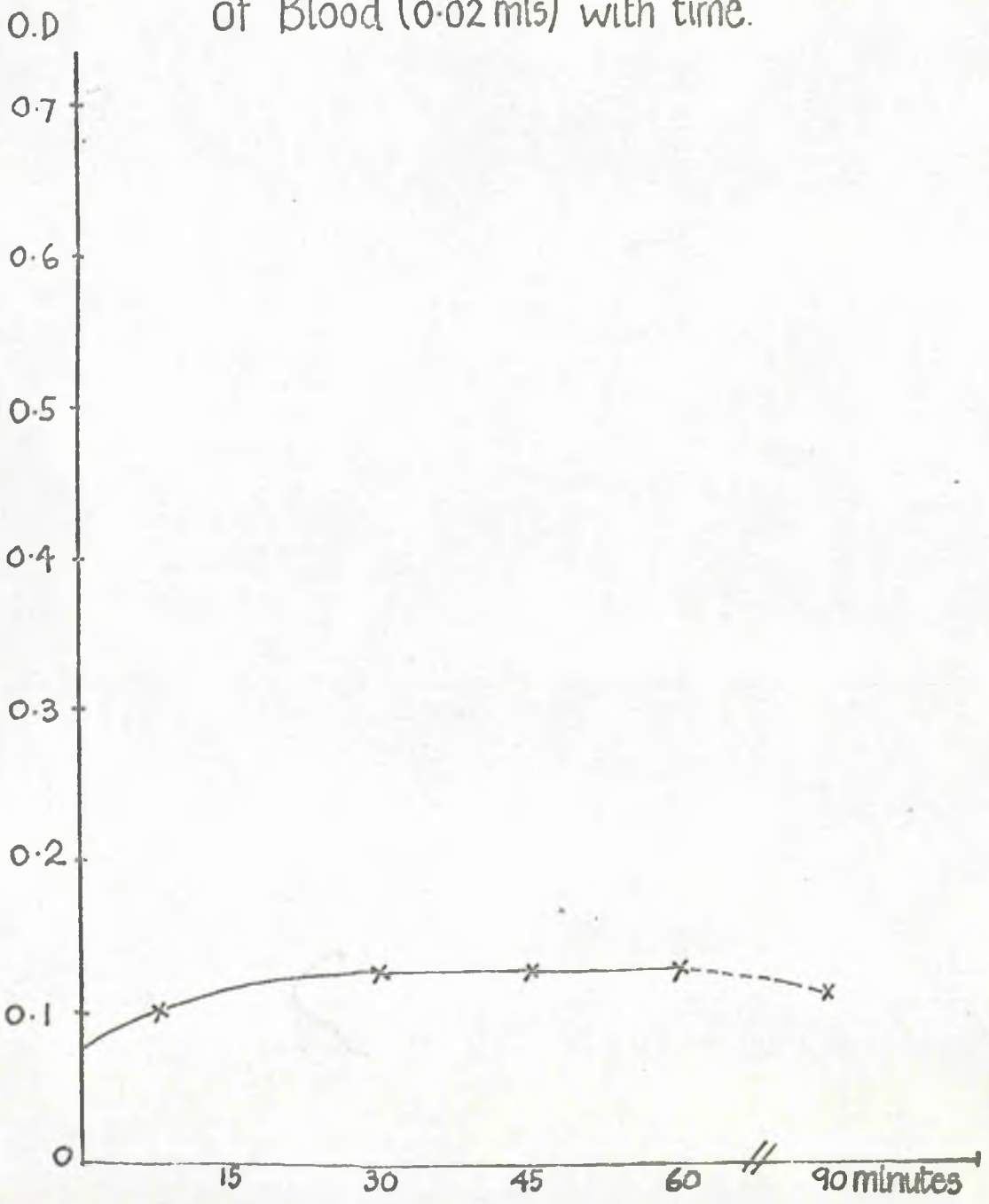
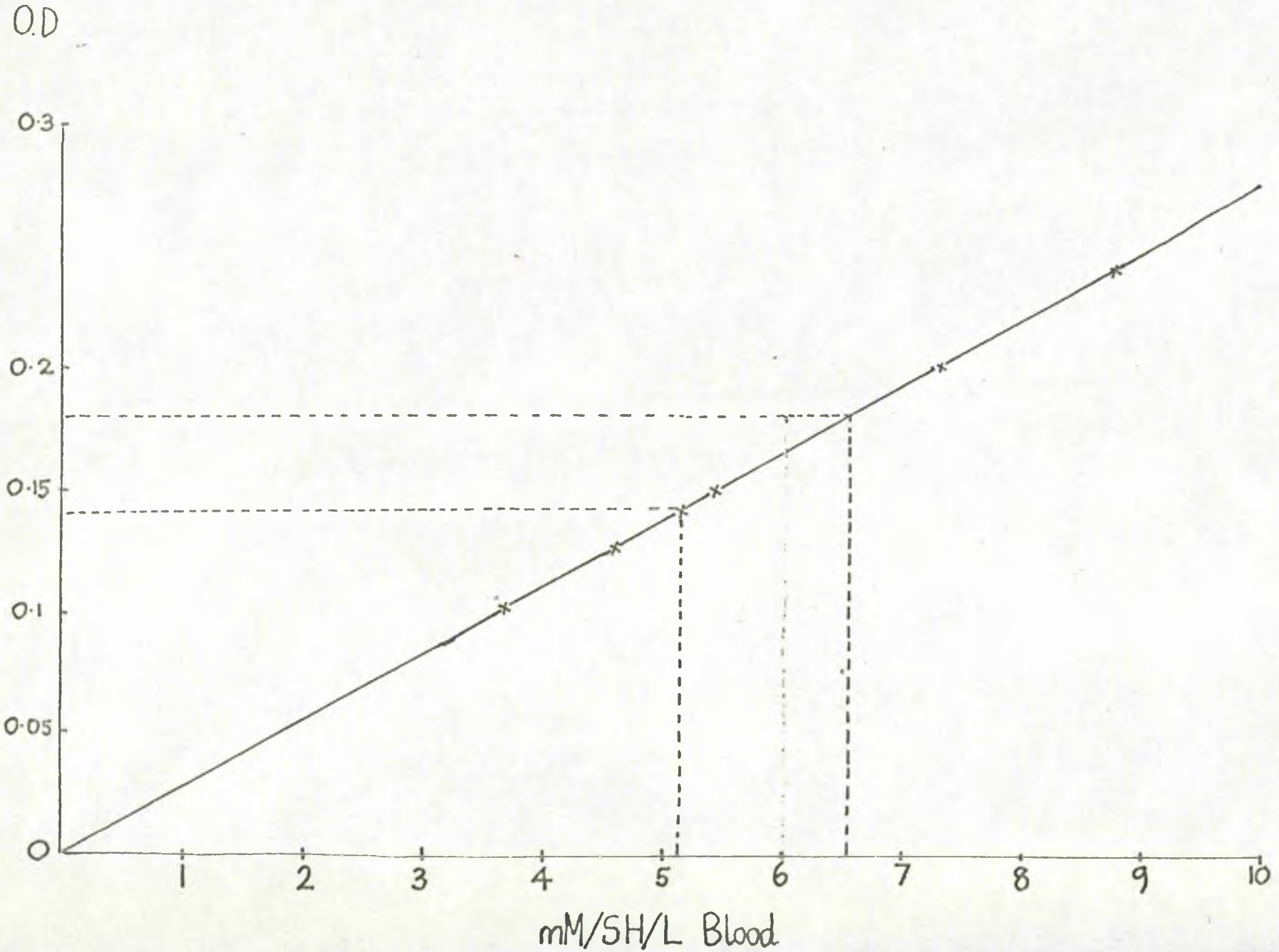


FIG. 2B.4 Calibration of Blood (0·02 mls) sulfhydryl groups. @ 420 mμ.



This experiment was repeated with two different fresh blood aliquots. These gave higher values of 5.15 and 5.47 mM/SH/L.

Fig. 2B.4 shows the calibration of blood - SH using the DTNB method. The results of three random female blood samples are

O.D.	mM/SH/L
0.125	4.6
0.14	5.15
0.1475	5.47

The dotted lines on the graph indicate maximum and minimum values of Ellmans. The above figures are within the minimum range of this worker. Mean female values are always somewhat less than males.

The effect of Urea on Whole Blood Fig. 2B.5

An equal volume of 8 M urea in phosphate buffer (0.1 M) pH 8.0 was equilibrated with an equal volume of blood, and divided into two moieties

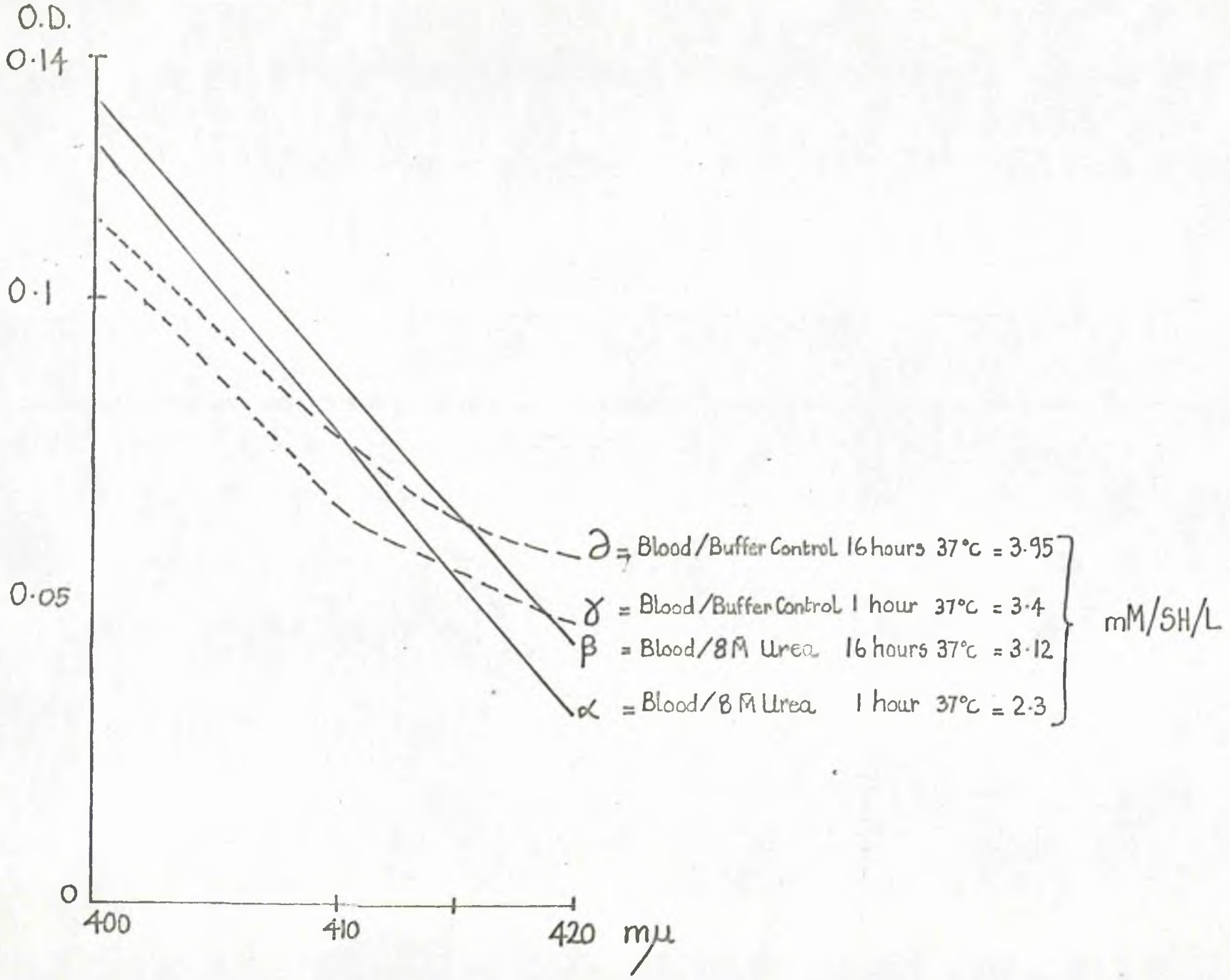
1) One fraction was kept at 37°C in a water bath for 1 hour, the other

2) was kept for 16 hr. under the same conditions.

As a control the same blood was equilibrated with an equal volume of phosphate buffer alone and treated as above for temperature and duration.

The results are seen in Fig. 2B.5. No change was observed at intervening times of 4, and 8 hours.

FIG. 2B.5 The effect of Urea on Whole Blood.



After correction for dilution low results of 3.12 and 2.3 mM/SH/L for the 16 and 1 hr Blood/Urea mixtures respectively were obtained.

Although Ellman has not commented on the effect of urea on blood he has shown that there was definitely no increase in plasma - sulfhydryl concentration in the presence of urea and in fact his figures show a small decrease

Plasma - SH - 0.133 mM/SH/L.

Plasma + 8.5 M Urea 0.131 "

Over 1 hour the percentage decrease in sulfhydryl concentration compared with control (blood + phosphate buffer) is 33% α/γ

Over 16 hours the percentage decrease is only 21% β/γ .

If the "denatured blood" (blood + urea) of 1 hour duration is compared with that of 16 hour duration, we find the following

% Increase in - SH from 1→16 hr = $\frac{3.12}{2.3}$ = 35.7%

The % Increase in - SH from blood + buffer control from

1→16 hrs = $\frac{3.95}{3.4}$ = 16.1%

Therefore, initially it appears there is a decrease in detectable - SH groups in blood denatured by urea but that over a period of 16 hours there is a detectable but very small rise in - SH groups from the denatured blood as compared with blood buffer control. This may well be within the limitations of this method and the significance of these findings will be discussed at the end of this section.

The mean of five individual readings is seen in Fig. 2B.5.

<u>The effect of increasing urea Molarity on reactive sulfhydryl groups of blood. Fig. 2B.6.</u>

Equal volumes of increasing urea molarity were added to separate but equal volumes of blood (0.02 mls) and the sulfhydryl groups assayed at 1 hr.

Control Blood Sulfhydryl = 5.2 mM/SH/L.

As the Urea Molarity is increased to 5 $\bar{M}$ the detectable-sulfhydryl concentration is 5.61 mM/SH/L - an increase of 8%. However 9 $\bar{M}$ urea produces a very marked decrease in sulfhydryl groups i.e. 5.04 mM/SH/L. Compared with the 5 $\bar{M}$ value this amounts to a loss of 10%.

Compared with the control value (5.2) this 9 $\bar{M}$ figure amounts to a total loss of - SH detectability of 3%.

<u>The effect of storage of blood on sulfhydryl reactivity.</u>

Blood was stored at 1-2^{o}C for 8 days. The sulfhydryl concentration was taken daily and four separate determinations done. Fig. 2B.7 shows the mean values. It is interesting that the graph pursues a similar pattern to the graduated denaturation by urea as above.

The first 4½ days show a gradual increase after an initial decrease probably partly due to mild denaturation but mainly to the mild haemolysis that takes place over this period. After 4½ days there is a dramatic fall to 3.3 mM/SH/L and for 2 days it appears to stabilise at this figure. It is worth

FIG. 2B.6 The effect of increasing Urea molarity on reactive sulfhydryl groups of blood (0.02 mls)

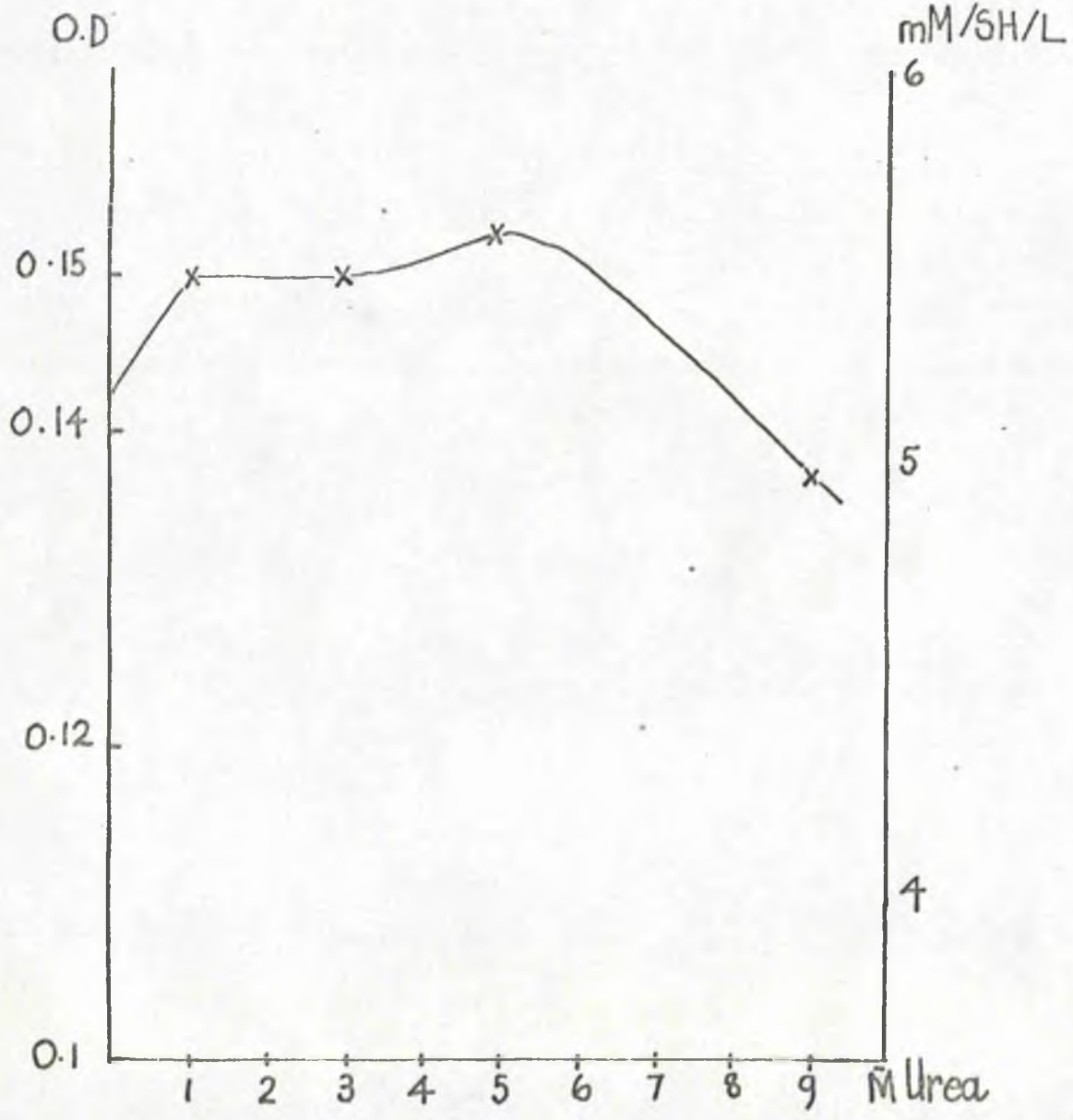
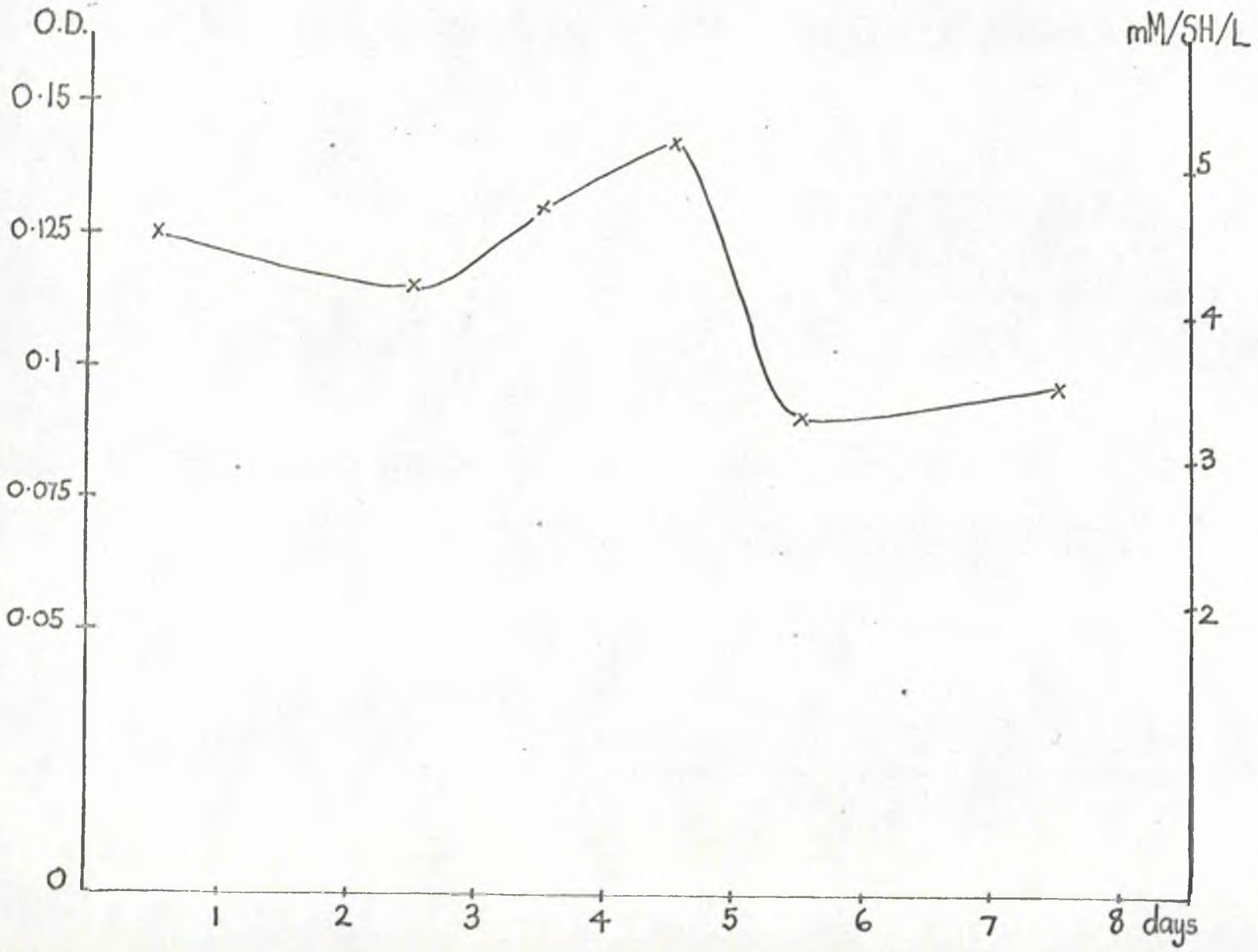


FIG. 2B.7 The effect of storage on Whole Blood -SH groups.



speculating whether there would be another significant drop after these 8 days, rather indicative of stepwise denaturation.

Denaturation of blood by organic solvents. Since many organic solvents are ideal denaturing agents the opportunity was taken of comparing Acetone, Ethanol, Dimethylformamide and methylethyl ketone.

Acetone 'denaturation'. 9 mls 40% Acetone were added to 1 ml phosphate buffer 0.1 M pH 8.0 and 0.02 mls fresh blood, and thoroughly but gently mixed.

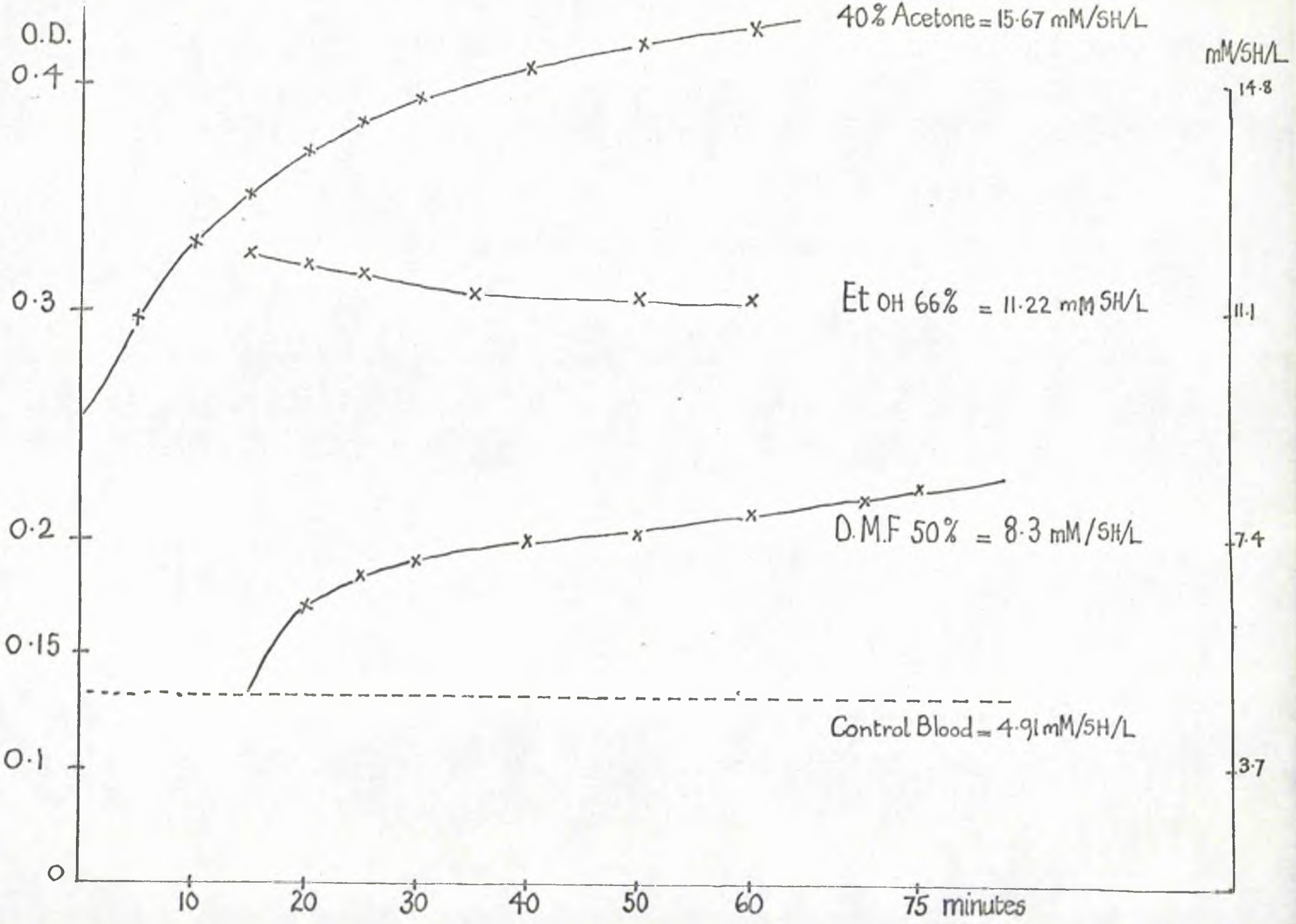
0.02 mls fresh standard DTNB solution was added 5 mins. later and the absorption curve plotted, Fig. 2B. 8.

This was repeated on another specimen of blood. The plots are remarkably constant and take at least an hour for maximum absorption to be obtained. At 60 mins. the λ max is equivalent to 15.7 mM/SH/L. This is an increase of over 300% of the amount of coloured anion normally produced by water alone (i.e. control) 4.91 mM/SH/L.

This experiment was repeated in duplicate with 66% Ethanol and 50% Dimethylformamide. Both mimicked acetone in the sharp rise in absorption over the first 10-20 minutes and the prolonged flattening out of the sigmoid curve over the next 60 minutes. The - SH values at λ max. were 11.22 and 8.3 mM/SH/L respectively.

In a second experiment an equal volume of 40% acetone was mixed with an equal volume of blood and carefully mixed. A 0.02 ml and 0.04 ml aliquot was taken and mixed separately in

FIG. 2B.8 Denaturation of blood by organic solvents



9 mls water and 1 ml phosphate buffer as in the standard experiment.

Exactly the same results were obtained as previously with λ max. being reached by 25 minutes in all cases. Absorption at 410 and 400 mu showed identical changes, fig. 2B.9.

The corrected sulfhydryl concentration at 420 mμ for the 0.02 ml aliquot was 8.8 mM/SH/L this represents only an 80% increase over the control.

The concentrations of the solvents used were the maximal permissible as in experiment 2B.8.

Above 40% Acetone flocculation of the protein ensues.

66% < Dimethylformamide causes precipitation

70% < Ethanol also causes precipitation.

Methyl ethyl acetone was tried as a denaturant but produced immiscible phases.

The Effect of different solvent concentrations on Denaturation.

Since it appeared that the most practical denaturing qualities of Acetone were 40% > and

Ethanol were 66% >

a table was compiled of the denaturing efficiency of various solvent concentrations.

The results are seen in fig. 2B.10 and tabulated below:

Denaturation Cascade

66% EtOH
V
50% EtOH
V
40% Acetone
V
30% Acetone
V
50% Acetone
V
60% Acetone

It must be borne in mind that these solvents were mixed with blood 50:50 v/v. So that the corrected dilution percentages are 33, 25, 20, 15, 25 and 30% respectively.

Corrected for dilution these give sulfhydryl values of

33% EtOH	8.8 mM/SH/L.
25% EtOH	8.6
20% Acetone	7.5
4 M Urea	4.4
15% Acetone	4.0
25% Acetone	3.96
30% Acetone	3.24

8 M Urea was added to the same batch of blood 50:50

FIG. 2B.9 The effect of 40% Acetone on the denaturation of fresh blood @ 410 and 420 mμ.

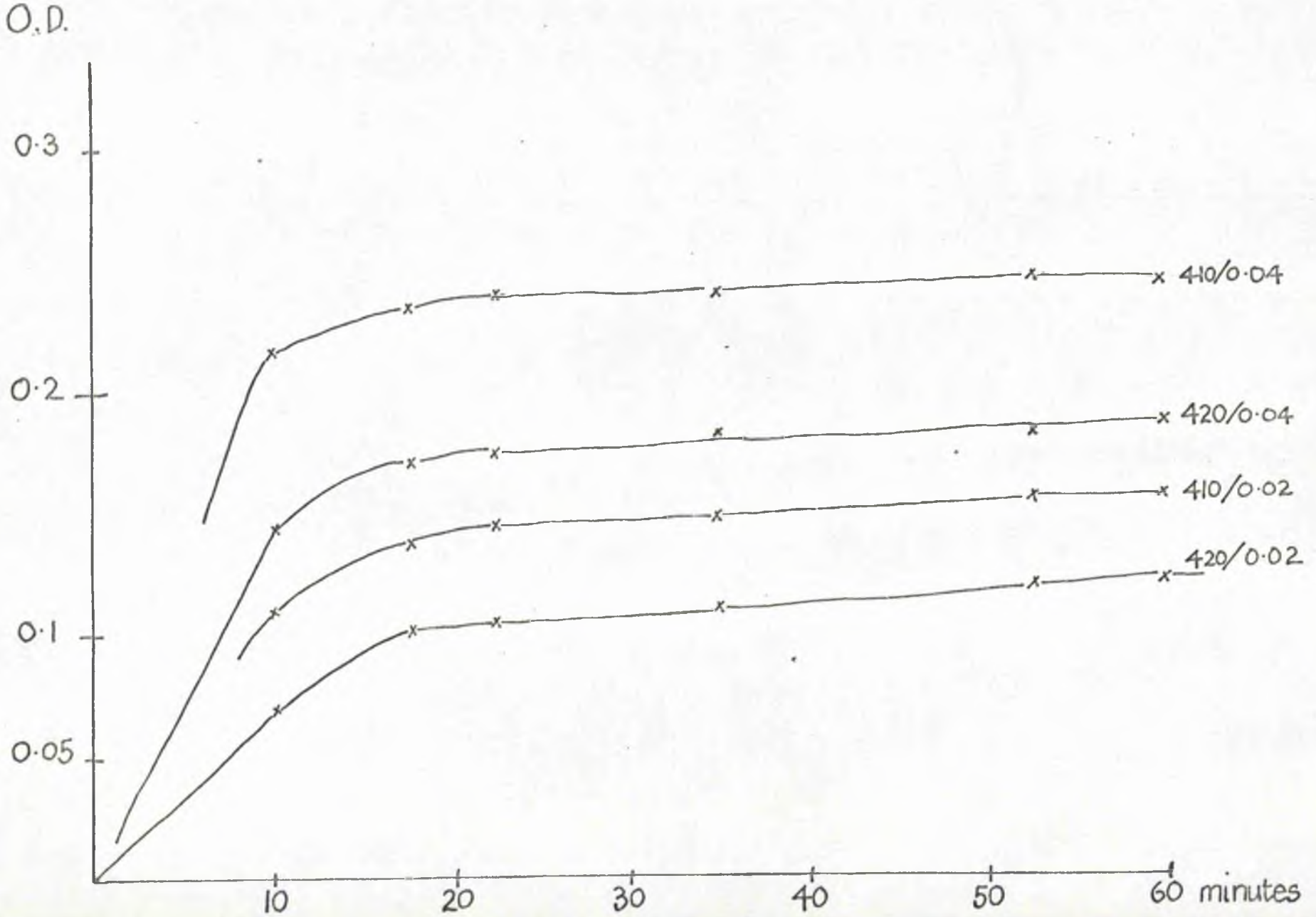
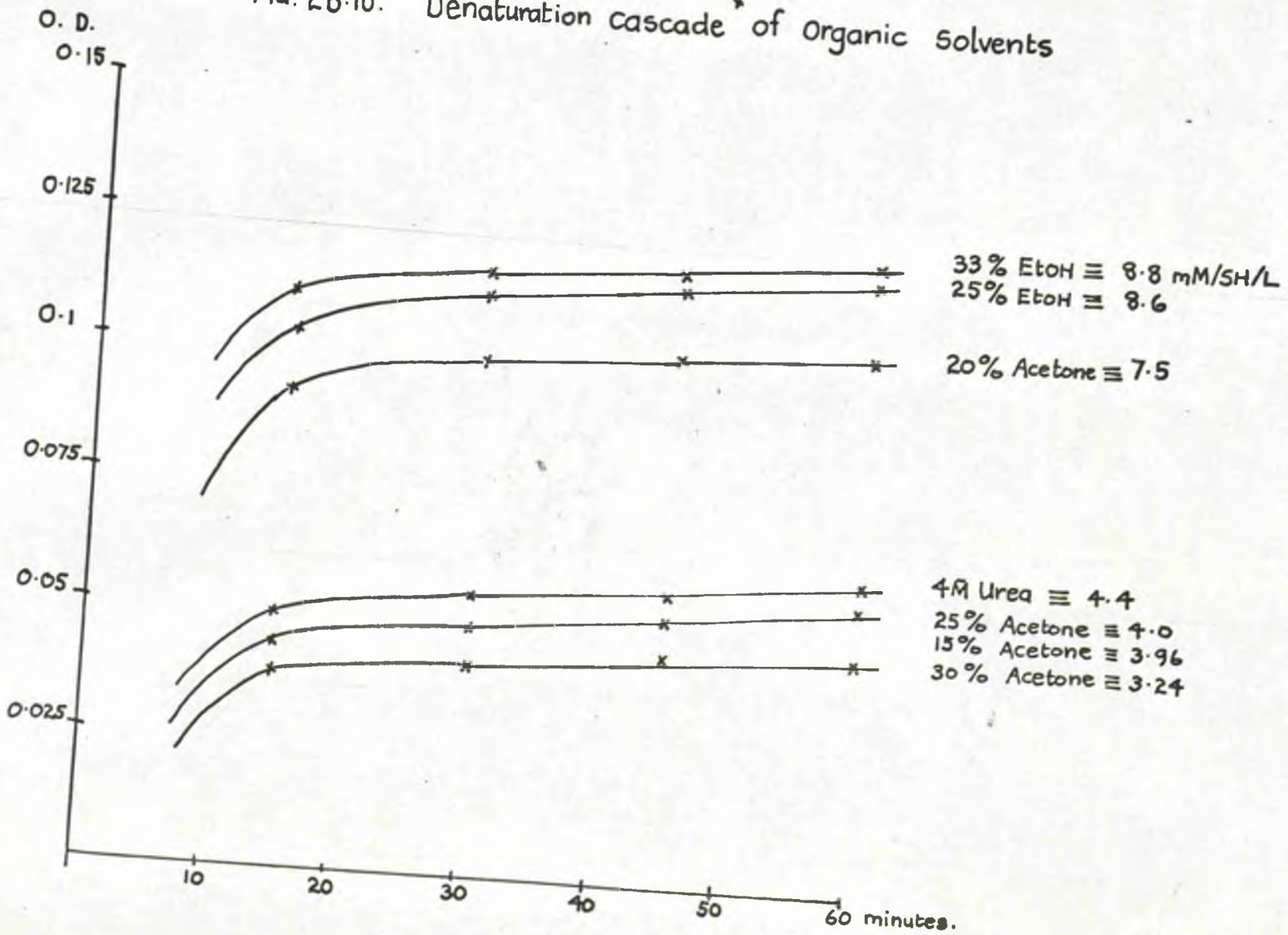


FIG. 2B·10. "Denaturation cascade" of organic solvents



v/v, thus giving a 4 M solution.

= 4.4 mM/SH/L.

The control blood value was 4.0 mM/SH/L (after dilution correction).

Comparing fig. 2B.7 and fig. 2B.8 it is clearly seen that the results of 20% Acetone (7.77) are exactly half of these at 40% (15.67 mM/SH/L). This is completely fortuitous and the only conclusion one can draw is that at these particular concentrations and 15% Acetone the sulfhydryl liberation from protein appears to have a linear relationship.

As can be seen from fig. 2B.9 however it is clear that other acetone concentrations certainly do not have a linear relationship. It is surprising in fact to see that 25% + 30% Acetone in fact give results below the control.

Other points will be mentioned in the discussion regarding acetone.

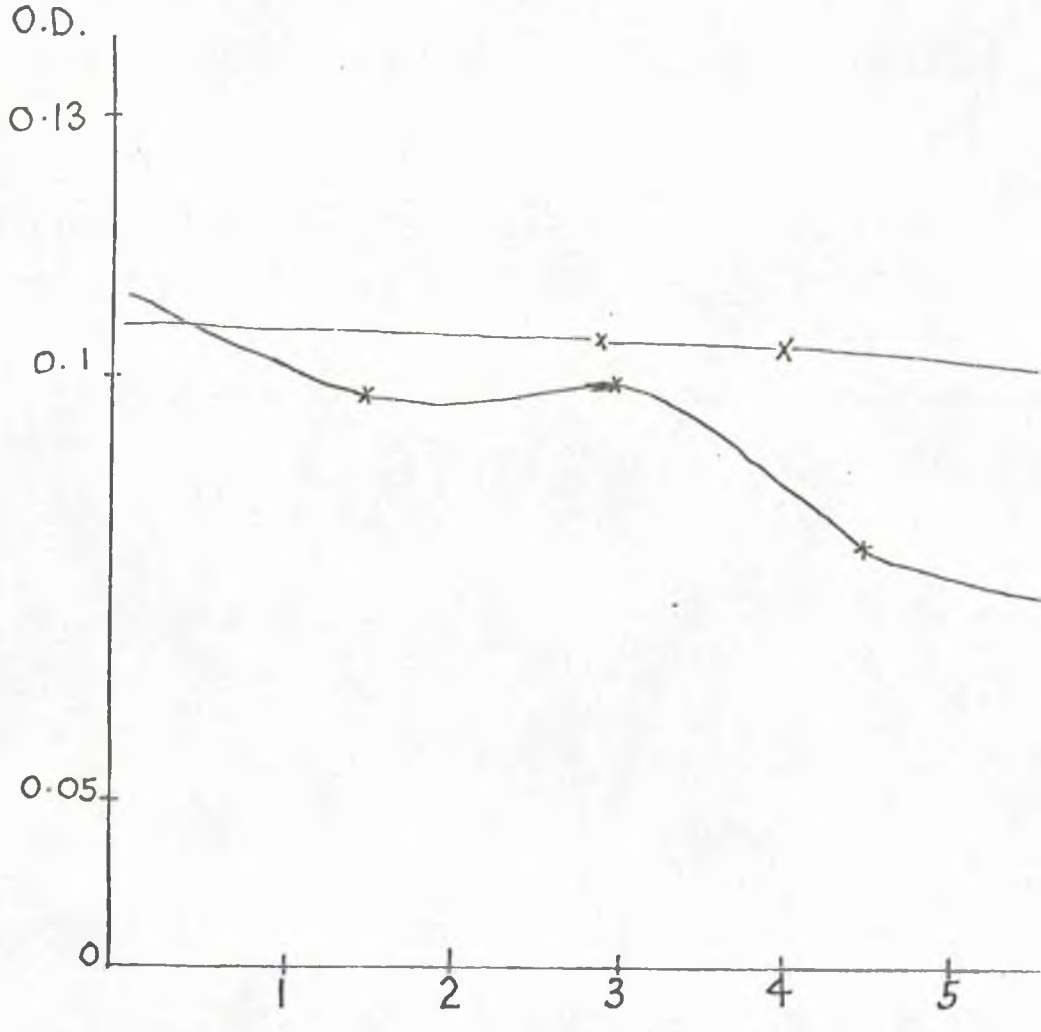
It would appear that Ethanol on the other hand does follow a linear relationship from the results of fig. 2B.7 and 2B.9.

EtOH 66% = 11.22 mM/SH/L

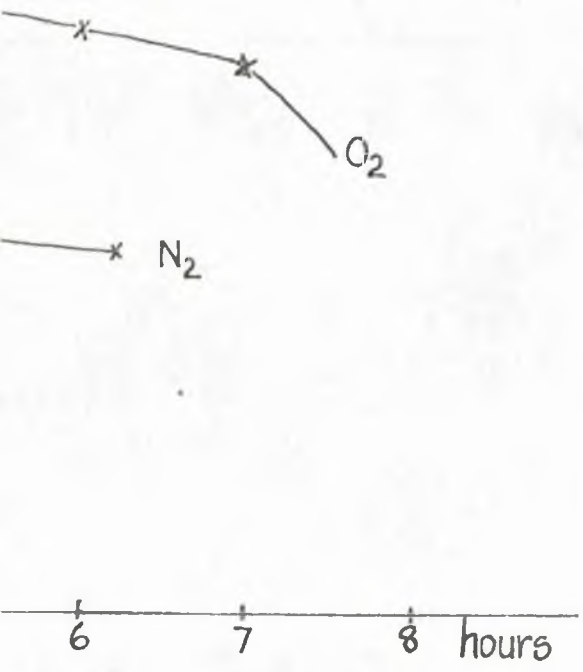
33% = 8.6 "

25% = 8.6 "

FIG. 2B.11 The effect of the prolor
of oxygen and nitrogen th



iged bubbling
nrough whole blood @ 420mμ



The effect of prolonged bubbling of nitrogen and oxygen through blood.

As in the maleimide section fresh, siliconed, heparinised blood was diluted with 12% isotonic saline and both gases were bubbled separately through 34 mls of the diluted blood for 7½ hours approximately.

0.02 ml aliquots of blood were removed at hourly intervals and immediately analysed by the DTNB method. The results are seen in Fig. 2B.11.

Compared with the lucid results obtained with maleimide, these results are rather equivocal. The effect of oxygenation does produce a fall in detectable sulfhydryl groups but this is very gradual for 7 hours when the fall becomes more abrupt. This effect was similar to, but more mild than the results obtained with maleimide.

The nitrogen effect.

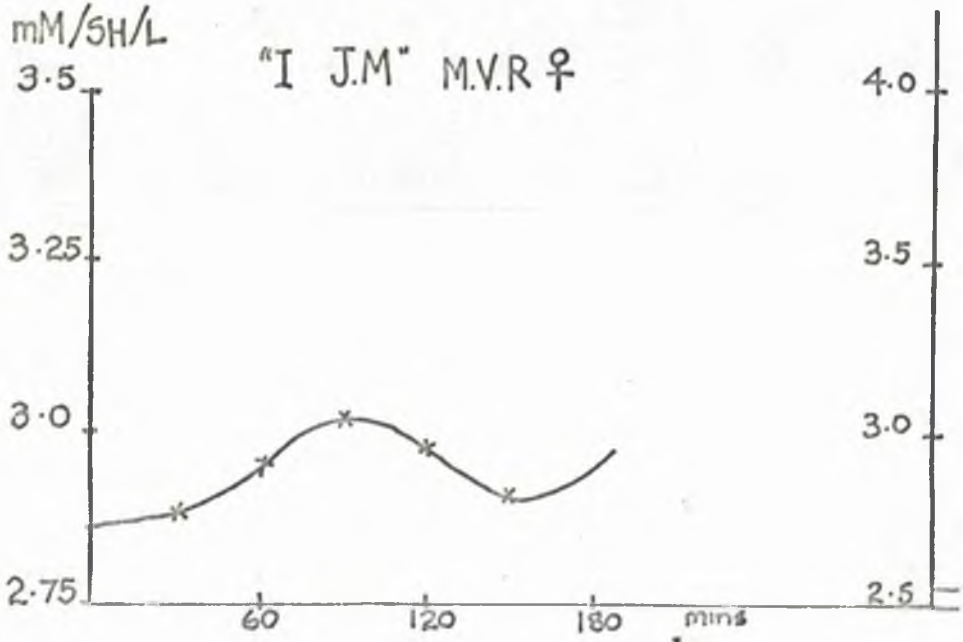
Instead of "preventing" loss of sulfhydryl groups by oxidation - as is suggested with the maleimide results, this curve shows a definite decrease from the control value; 4.05→ 2.68 mM/SH/L.

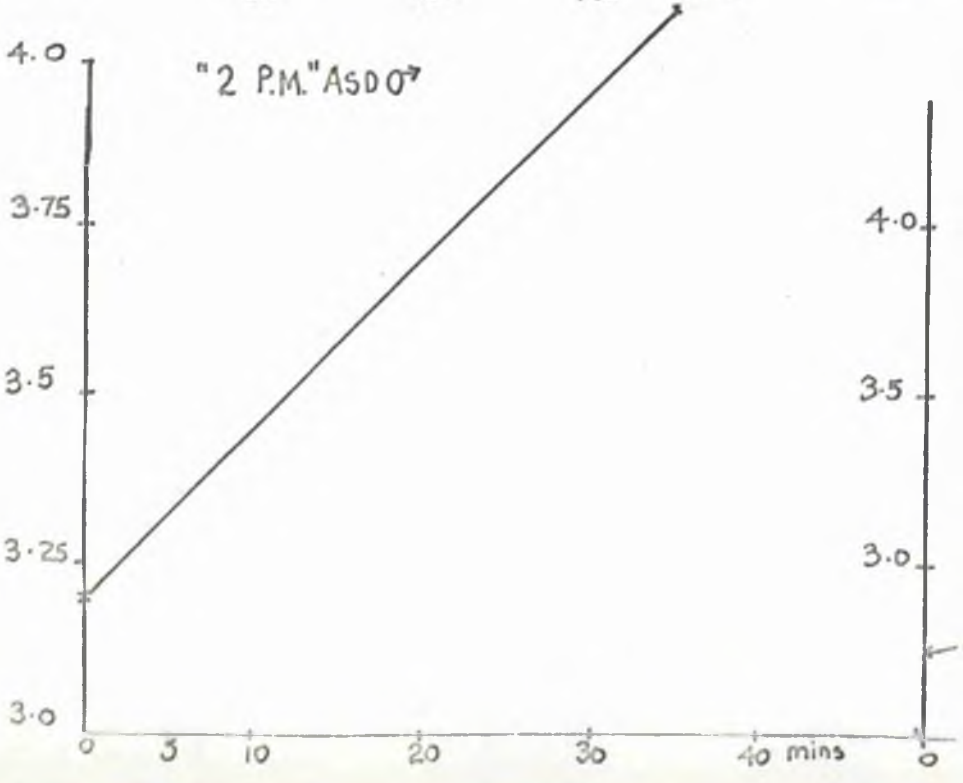
Changes in - SH of blood during operations.

The increase in the - SH content of operations is seen in figs. 2B 12-2B 16. The final values range between 5-10% above the control values.

In the Melrose experiment there is a vast increase in - SH content - but only after 3 hours on the closed circuit. The implications of these changes together with those in the

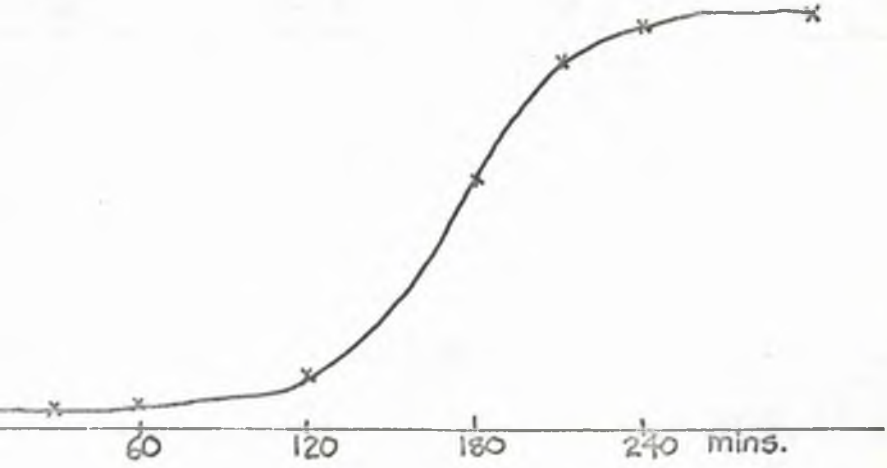
FIG. 2B·12 DTNB : Blood -SH (0·





02 mls.)

"4. M.Ch" A.V.R. ♀



"3 B.K" ASD/VSD ♂

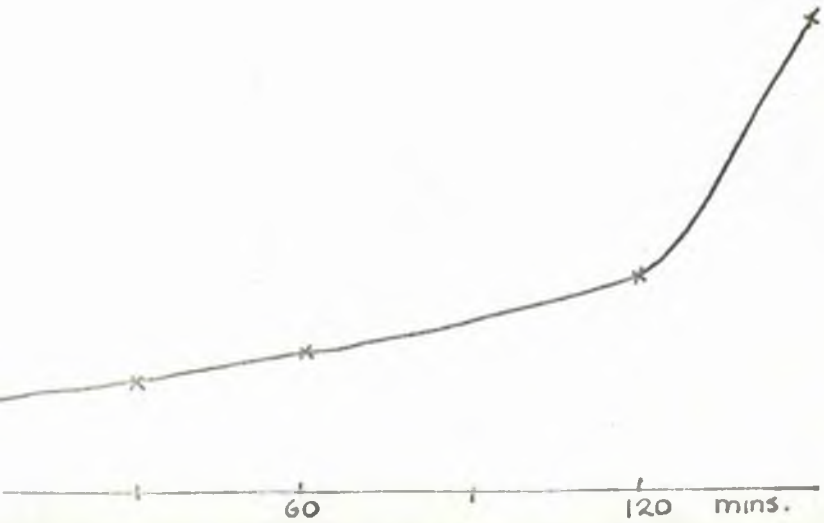


FIG. 2B·13 DTNB: Blood -SH (0·02mls)

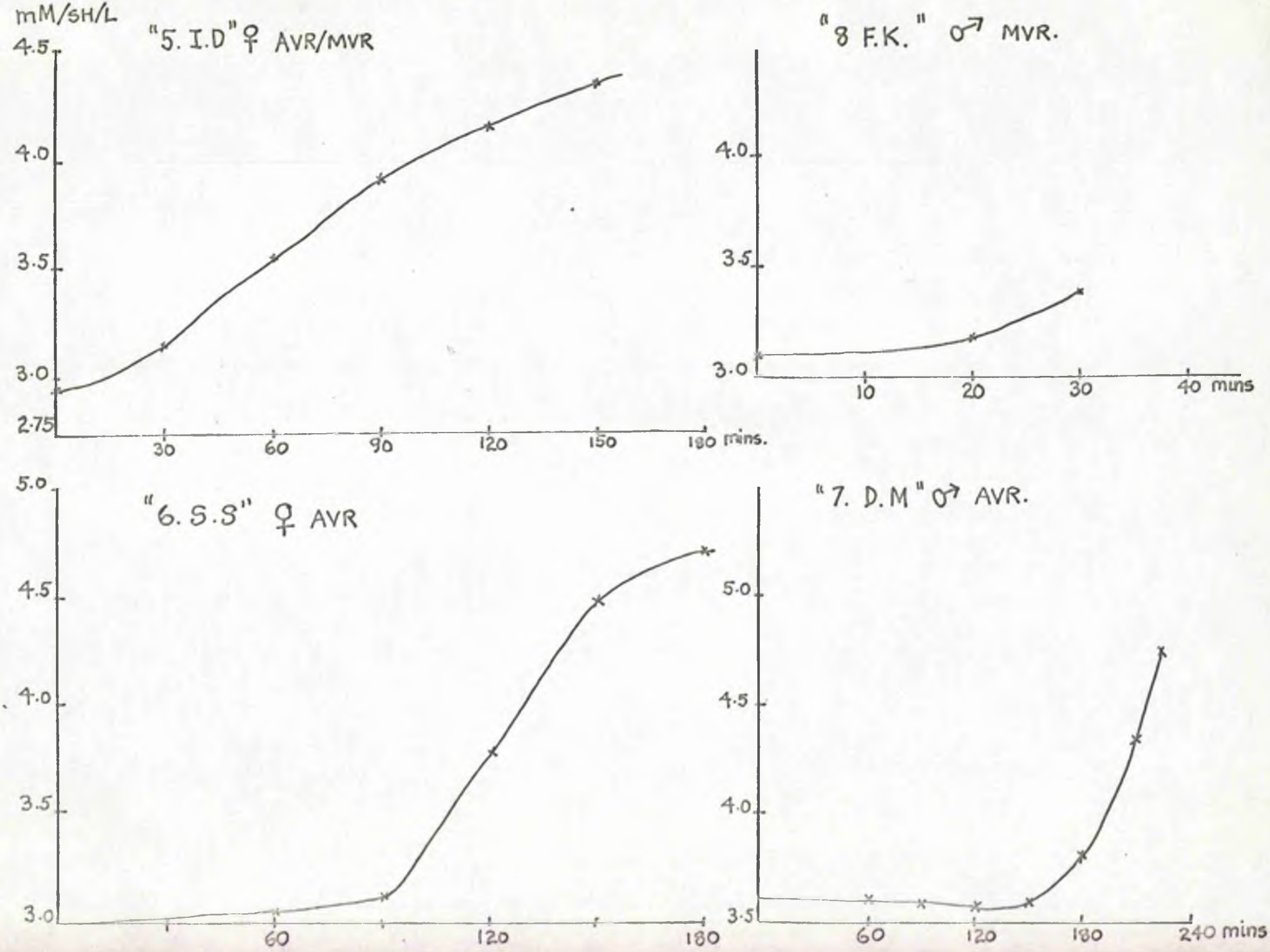


FIG. 2B·14 DTNB: Blood -SH (0·02 mls)

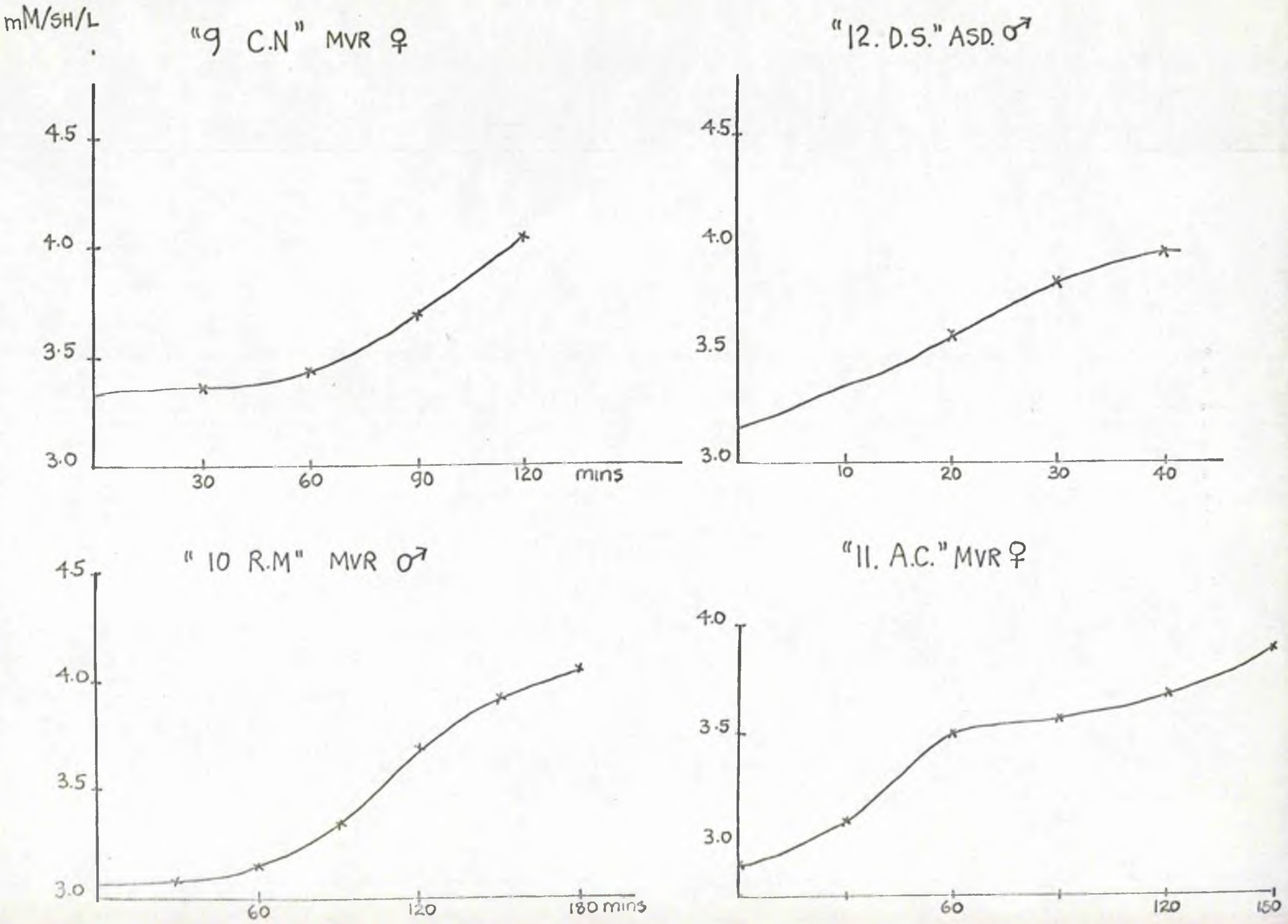
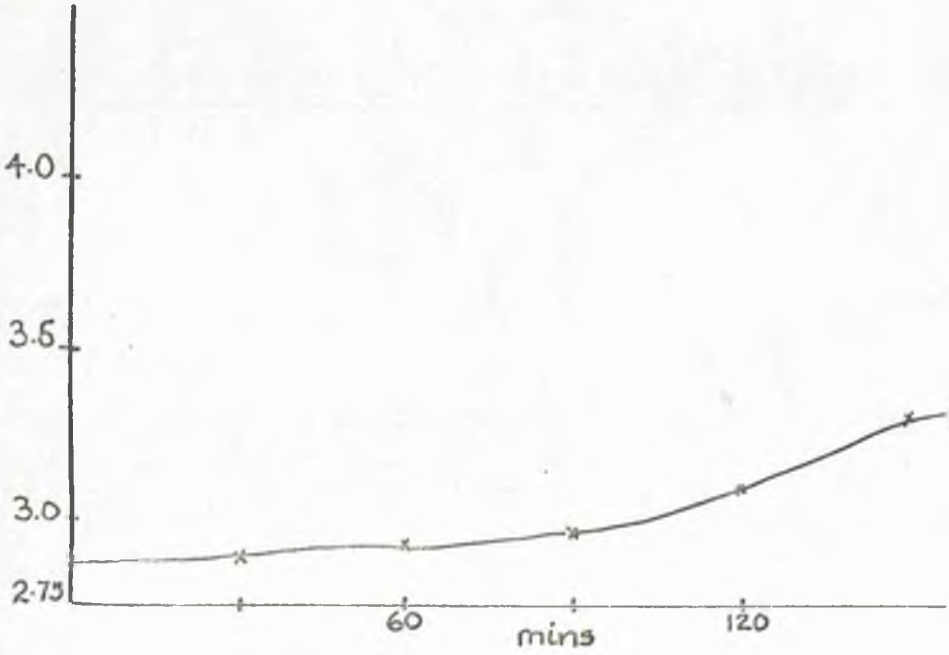


FIG. 2B·15

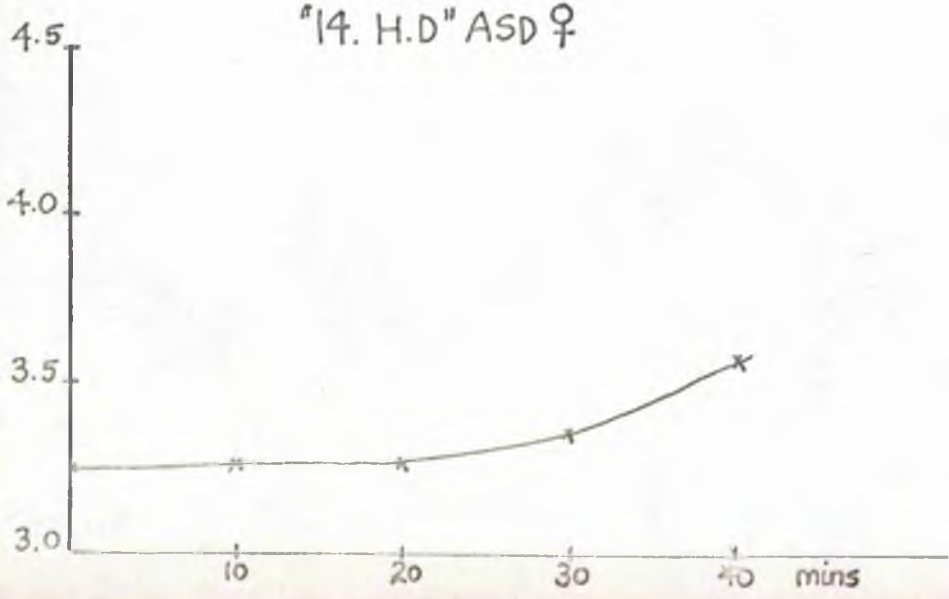
DTNB: Blood - SH

mM/SH/L

"13. R.D." AVR ♂

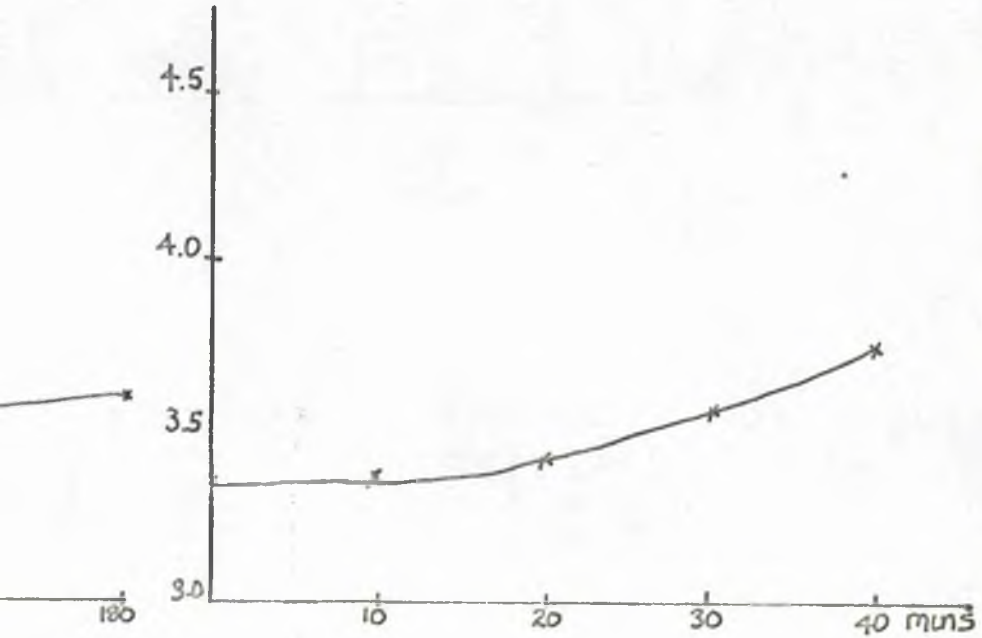


"14. H.D" ASD ♀



(0·02 mls)

"16. K.R" ASD ♂



"15 J. McC"

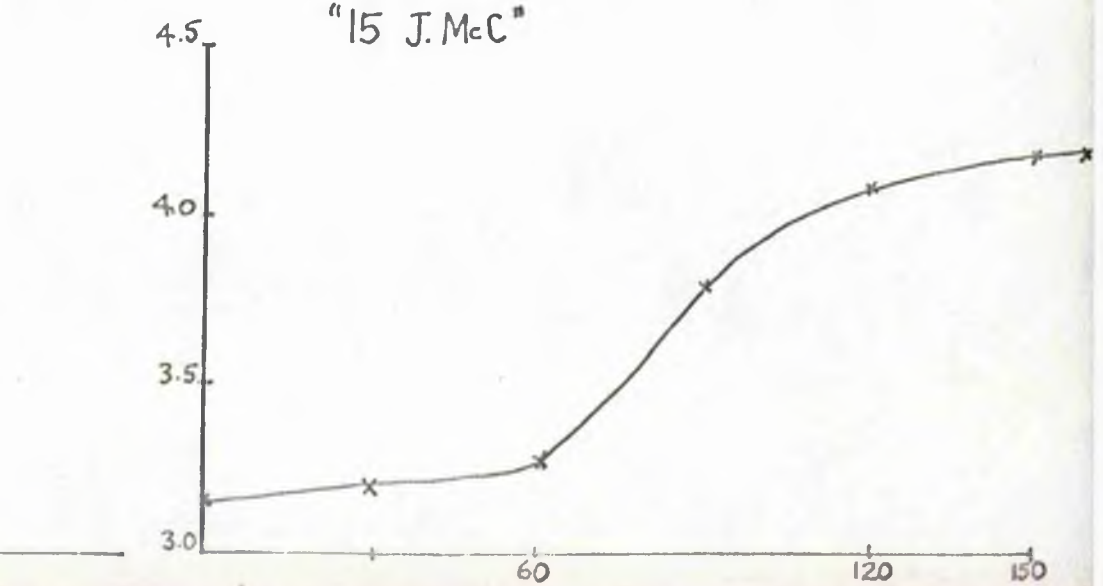
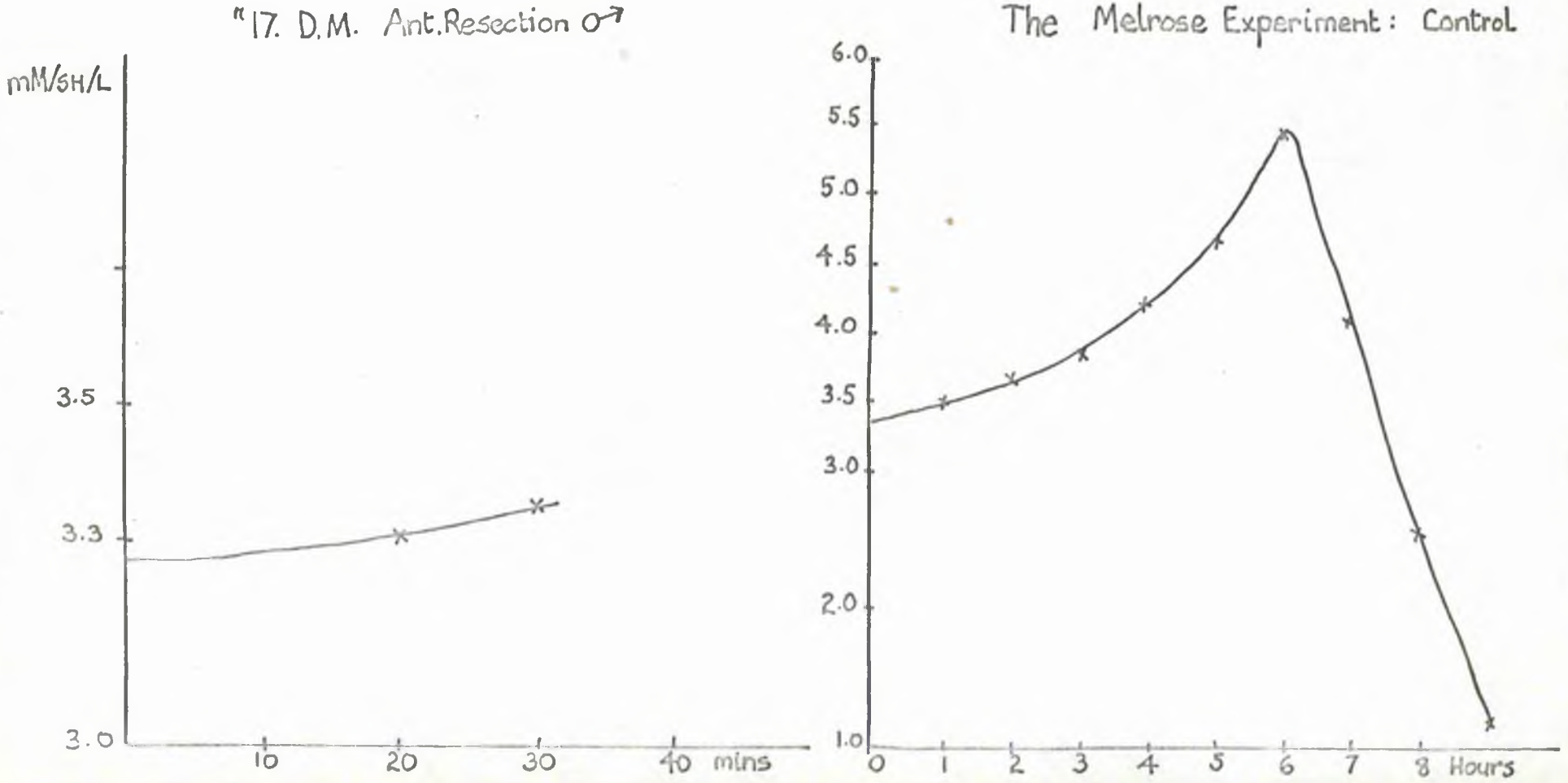


FIG. 2 B·16 D.TNB: Blood-SH (0·02 mls)



previous oxygenation experiment are discussed below.

The individual - SH values from patients and the Melrose experiment may be seen in Appendix 2.

Discussion.

In studying the effect of increasing Molar content ratios of urea on blood it is seen that after 5 $\bar{M}$ there is a decrease in sulfhydryl detection. It might be postulated therefore that 1-5 $\bar{M}$ Urea exposes some normally hidden sulfhydryl groups which may be detected by DTNB, but that increasing the urea concentration from 5-9 $\bar{M}$ produces further conformational change which "inadvertently" makes previously detected - SH groups unavailable or unreactive.

It may also be suggested that the change in sulfhydryl detection is due to several other reasons.

1) That the destruction of - SH groups occurs by factors unknown in excess of the rate at which denaturation is exposing these groups for reaction with DTNB.
2) Oxidation of the sulfhydryl groups to disulphide is a possibility depending on the oxidative properties of a particular denaturing agent. In this respect it is worth mentioning the action of acetone on blood which turns the latter brown, suggesting oxidation of Haem iron $\longrightarrow$ ferric state. Acetone clearly makes - SH groups more reactive.

Ellman, 1959, has shown that Haem in the ferric state (λ max = 578 mμ) is inversely proportional to Haem in the

ferrous state (λ max = 412 mμ)

i.e. A_{578} = K' A 412 mμ.

or in other words

$$\text{Haem } Fe^{+++} + S_2 \rightleftharpoons \text{Haem } Fe^{++} + -SH$$

If however acetone is responsible and does indeed oxidise the ferrous Haem to Fe^{+++} how can this possibly be reconciled with the fact that more sulfhydryl groups are being made available for DTNB detection by reduction of disulphide. Since both these facts are favouring opposite reactions e.g.

$$-SH + \text{Haem } Fe^{++} \longrightarrow \text{Haem } Fe^{+++} + S_2 \quad \text{(oxidation)}$$

$$\text{"} \quad \longleftarrow \quad \text{"} \quad \text{(reduction)}$$

There are two possible sources of disulphide which the Haem Fe^{+++} might reduce.

1) The globin portion of the molecule

2) Disulphide links in other erythrocyte proteins.

Ultracentrifugal analysis of plasma proteins will be considered in a later section. The 4S fraction of plasma proteins includes Albumin (90%) and α_2 globulins (10%). The latter contain haptoglobin, caeruloplasmin and many enzymes. This 4S sedimentation constant is decreased to 1.755S upon the addition of acetone to blood. This is of course equivalent to an appreciable decrease in molecular weight and apropos of this it follows that many hitherto undetected or unreactive sulphydryl groups are made available for DTNB detection.

This work agrees with Ellman that Acetone does increase the coloured anion some 200% (compared with control) when mixed

with Blood and DTNB, but certainly it fails to show the disappearance of the additional sulfhydryl groups which Ellman indicates takes place after 20 mins. with reaction with DTNB. Indeed the foregoing graphs show consistently that maximum absorption is reached after 20 mins. reaction and remains at this level for at least 40 minutes.

Ellman shows that D.M.F. (dimethylformamide) in the presence of nitrogen also gives this 200% increase. Although nitrogen was not used in the foregoing experiments D.M.F. was used alone with Blood and DTNB (Fig. 2B.7) and showed an increase of 66 $\longrightarrow$ 76% (8.3 $\longrightarrow$ 8.8 mM/SH/L).

It is interesting that the increase in reactive sulfhydryl groups of blood in the presence of urea is minimal. A maximum of only 8% being found. Ellman's figures on <u>plasma</u> show no detectable increase in the presence of 8.5 $\bar{M}$ Urea nor in fact in the presence of 50% Acetone - this appears to be inexplicable other than the fact that normally unreactive sulfhydryl groups are not exposed by these reagents and possibly considerably resistant to exposure.

As regards the change of sulfhydryl groups with storage fig. 2B.6 would suggest that stepwise denaturation may well be taking place rather than in a progressive linear way. The fact that the sulfhydryl detection is <u>decreasing</u> confuses the issue as to whether denaturation is indeed taking place at all. It could be proposed that, as mentioned above, the reactive sulphydryl groups made available by both haemolysis and

denaturation are oxidised or destroyed at a rate greater than their availability to react with DTNB.

Urea as a denaturing agent is of interest as it appears to show the phenomena of reversibility. It is odd to suggest that in the presence of increasing urea concentration in blood that in fact denaturation does not appear to be taking place but undergoing reversal to the native protein. In Fig. 2B.6 this change is minimal. The decrease in - SH groups however is most likely due to oxidation and/or destruction.

As mentioned above Ellman showed that DTNB detected no increase in plasma sulfhydryl groups in the presence of urea or acetone. It thus suggests that although - SH groups are certainly present in plasma (e.g. albumin), they are not readily available even in the presence of common denaturing agents. It is reasonable therefore to assume that oxygenation of blood also would not increase - SH of plasma. If it does then simple oxygenation would thus appear to have stronger denaturing ability than 8.5 M Urea and 40% Acetone, which would appear to be unlikely. The only way to solve this problem is to oxygenate a relatively large volume of plasma for several hours and then assay the - SH content. This would have to be done on relatively fresh plasma which is considerably expensive.

Since DTNB shows no increase of blood - SH groups in the presence of urea and acetone it is unlikely for N-Ethyl maleimide to show any changes either.

The effect of bubbling oxygen and nitrogen through

separate samples of blood is of great interest for in this experiment there is not the dramatic change which is seen in the corresponding maleimide assay. This is difficult to explain - unless one suggests that in blood, nitrogen does not have such a protective effect on - SH content as it appears to have in plasma. This may be due to oxidising and degradative mechanisms which are relatively absent in plasma.

It is also possible that maleimide and DTNB react partially with different sulfhydryl groups. It is just possible, although unlikely that some of these groups are normally unreactive with DTNB. It is unlikely that nitrogen interferes with the sulfhydryl DTNB reaction.

Although oxygenation of this small volume of blood and the consequent assay of - SH in blood and plasma aliquots by DTNB and NEM respectively, shows a gradual decrease over several hours it is very interesting to see that in the Melrose experiment in which a large volume of blood (1500-2000 mls) was being oxygenated that the - SH markedly increases over the first 6 hours, after which there is a dramatic fall. This suggests that in this large 'perfusate volume' the - SH groups liberated are protected from destruction or other loss possibly by buffering action or certain inhibitors of - SH destruction/alteration present. After 6 hours it would appear that this protective mechanism fails and rapid loss of these groups therefore occurs.

It is of importance although not surprising to see that the DTNB results from operation blood samples give virtually

identical results with the NEM assay on the respective plasma aliquots. This indicates that both reagents were detecting the same reactive - SH groups.

CONCLUSION.

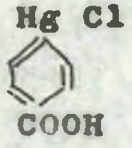
1) DTNB is a specific - SH reactant readily available for routine serial estimation of blood samples.
2) The effect of urea on blood gave equivocal results. Its denaturing effect appeared minimal.
3) Storage at 1-2°C for 8 days produced partial loss of reactive sulfhydryl groups. Thus analysis as soon as possible after collection is of great importance to prevent
   (a) Further haemolysis occurring, and
   (b) Loss of these groups.
4) 40% Acetone produced apparently 100% denaturation (15.7 mM/SH/L) since no other common denaturing agents gave - SH concentrations in excess of this.
5) The effect of varying concentrations of Ethanol and Acetone have been described.
6) The effect of gassing (O_2 + N_2) blood gave equivocal results, probably due to only 30 mls of blood being used.
7) The - SH content from operation blood samples showed an increase of 5-10% over the control values.

SECTION 2C.

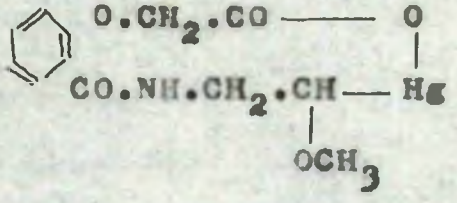
p-chloro mercuri benzoic acid (PCMB)

INTRODUCTION.

The use of organic mercurials as specific reagents for sulfhydryl estimation are well known. There are three alternatives.

1) Phenyl mercuric compounds  e.g. PCMB.

2) Mercurated allylamides. In this group is the well known derivative - a potent modern diuretic - Mersalyl.



Opening of this ring system and the addition of Cl^- or OH^- results in greatly increased solubility.

3) Alkyl mercuric compounds. These include methyl mercuric nitrate and methylmercuric iodide. The major disadvantage of these substances is their high toxicity.

For studies on denaturation of the blood p. chloromercuri benzoate was used (formula I above). The great advantage of organic mercurials is their monovalent reaction with sulfhydryl groups. One of the valences of mercury being occupied by a covalent bond with carbon.

In PCMB the carbon mercury bond is very stable even in acid. The other advantages of PCMB in sulfhydryl estimation are:-

1) The great affinity for - SH groups which enables PCMB to be effective under mild conditions and in low concentrations. It is also more likely to react with "latent" or partially unreactive - SH groups (due to stoichiometric position).
2) PCMB reacts with single - SH groups, and
3) reacts with these groups that appear masked to many oxidising agents.
4) It is a highly selective agent in that it combines with no protein group other than - SH.
5) The combination of PCMB with - SH groups is reversible by the addition of an excess of a simple mercaptan.
6) PCMB presents comparatively low toxicity.

<u>Disadvantages.</u>

1) In the presence of a halide, PCMB is poorly soluble.
2) It is difficult to obtain a high degree of purity.
3) In employing the titration technique in - SH assay it is a time consuming procedure when serial samples have to be analysed, especially as meticulous care must be exercised with each determination. There is a very real risk of - SH loss on standing at room temperature due to factors such as oxidation and degradation mentioned in "the introduction to sulfhydryl analysis" and elsewhere in this work.

The first problem mentioned above may be overcome by complexing the mercury with such anions as pyrophosphate or glycyl glycine, anions which are able to displace the chloride very readily.

The affinity of hydroxide ion for the mercury is far greater than that of the chloride ion.

PCMB solutions have therefore been prepared in the presence of sodium hydroxide, thereby making a mixture of p-hydroxy mercuri benzoate and p. chloro mercuri benzoate.

The second disadvantage, above, is overcome by regular titration with fresh standard glutathione (red).

Sulfhydryl Assay.

In the application of PCMB to - SH estimation the most practical method was the use of spectrophotometric titration. Before this is described, three other methods of - SH assay by PCMB are worth mention:

1) It has been shown by Ellman, (1958) that the disappearance of - SH groups using nitroprusside as indicator is by no means specific for these groups. In pure 'in vitro' analysis, - the total - SH groups only can be assayed. (Edelhoch, et al., 1953)

2) Colorimetric determination of excess mercurial. This involves solvent extraction, the risk of protein denaturation and is time consuming in the case of serial blood samples. See Hughes, 1949; Simpson, 1958 and Fridovich, 1957.

3) Amperometric titration (polarographic method) of excess mercurial, see Kolthoff and Harris, 1946.

Preliminary work with this method was done but due to logistics could not be continued. The basic theory of this

excellent method is thus:

Using a rotating platinum or dropping mercury electrodes, a current is passed through the reactant mixture (e.g. silver nitrate, ammonia, 0.25M, and protein solution to be assayed).

The silver nitrate when added first binds covalently with the protein only by the - SH groups. The number of silver atoms bound is then equal to the number of available - SH groups.

When silver ions are in excess, a diffusion current to the electrode (e.g. rotating platinum) is then observed and indicates the titration end point and therefore the number of silver atoms held by the protein.

Ingram, 1955, used polarography in the determination of available - SH groups in native and denatured haemoglobins from man and other sources. Silver nitrate and mercuric chloride being used in the titration.

For ease of reference, methods and results are given together in all the following PCMB titrations.

Methods.

The PCMB application to sulfhydryl determination in blood samples.

Principle:

The use of PCMB in - SH determination depends upon the increase in absorption at 250 mμ as a result of the formation of mercaptide. It is the only mercurial which can be applied in a practical quantitative estimation of - SH groups. Other mercaptides being relatively insensitive to determination of mercaptide formation in the useful spectrum.

PCMB was reacted with plasma and the increase in absorption noted. The procedure is carried out in the form of a titration - the PCMB being the titrant, - for two major reasons.

1) The proteins present absorb strongly at this wavelength; and this is overcome by appropriate blanks.
2) The Molar Extinction Coefficient Δ_{ϵ} differs from protein to protein.

The degree of reactivity of PCMB for - SH groups depends on such factors as pH, ionic strength and the presence of denaturing agents such as urea.

pH especially is of great importance since PCMB contains an ionised carboxyl group and has a high affinity for hydroxyl ions, - these tend to increase its reactivity as the pH is lowered.

Thus at lower pH's the total available - SH concentration may be more easily measured, i.e. reactive and unreactive (or less reactive). This is easily demonstrated when Haemoglobin is used.

Thus the reason for using Solutions A and B below.

The interest in exactly estimating the - SH content of haemoglobin is due to the following factors.

1) Haemolysis of blood occurs during open cardiac surgery.
2) Denaturation of protein gives rise to - SH liberation.

Therefore one immediate difficulty in assaying - SH in free plasma and/or blood is the question - to what degree is this increased - SH content due to haemoglobin and to what

degree plasma proteins.

At pH 7.0 samples were assayed at 250 mμ for reactive - SH estimation.

At pH 4.6 samples were assayed at 255 mμ for total - SH content.

Experimental

The standard stock PCMB solution was made up thus:- 8 mgms PCMB was dissolved in 1 ml of 0.04 sodium hydroxide (one equivalent) and made up to 25 mls with water.

Solution A. Usually 2 mls of stock PCMB was diluted to 25 mls with 0.05 M̄ phosphate buffer, pH 7.0, although departures from this procedure were used to give better intercepts in some titration procedures.

Solution B. 2 mls of stock PCMB was diluted to 25 mls with 0.033 M̄ Acetate buffer pH 4.6.

Standardisation of standards.

0.0162 gms glutathione (red) was dissolved with great care in 10 mls water. A 0.04 M̄ sodium hydroxide solution (1.6 g/L) was prepared.

Assuming glutathione to be 98% pure the molarity of its solution will be

$$\frac{16.2}{307} \quad \frac{98}{100} \quad x \quad 0.1 \text{ M̄ - SH} = 5.17 \times 10^{-3} \text{ M̄}$$

In titration of PCMB with this glutathione solution the molarity of the former will be equal to

the Molarity of GSH x $\frac{\text{Volume of GSH at end point.}}{3}$

$$= 0.00517 \times \frac{0.0305}{3} = 5.273 \times 10^{-5}\ \bar{M}\ \text{PCMB}$$

using 3 mls of the PCMB solution A.

Due to the relatively poor solubility of PCMB, the importance of its standardisation is shown in the following comparison of molarities derived theoretically and by titration.

Theoretical molarity of PCMB

8 mgms of PCMB in 25 mls ($NaOH$: H_2O/1: 24 mls as above)

$= 0.32$ g/L

$\therefore$ Molarity of stock solution $= \frac{0.32}{357.18} = 8.96 \times 10^{-4}\ \bar{M}$

Solution A

2 mls stock PCMB was diluted to 25 mls with phosphate buffer - 0.05 $\bar{M}$

$\therefore$ theoretical molarity $= \frac{8.96 \times 10^{-4}}{12.5} = 7.168 \times 10^{-5}\ \bar{M}$

The Molarity by titration has been seen to be

$5.273 \times 10^{-5}\ \bar{M} = 0.0188$ g/L

Therefore the correct PCMB molarity (titration) as a percentage of the calculated

$$= \frac{5.273 \times 10^{-5}\ \bar{M}}{7.17 \times 10^{-5}\ \bar{M}} \times 100 = 73.55\%.$$

Therefore it is essential that PCMB solutions, preferably fresh, are standardised prior to use in - SH assay work. This decrease of 26% approximately from the expected molarity is due to the partial insolubility of the organic mercurial.

FIG. 2c.1 The standardization of Glutathione by PCMB @ 250 mμ.

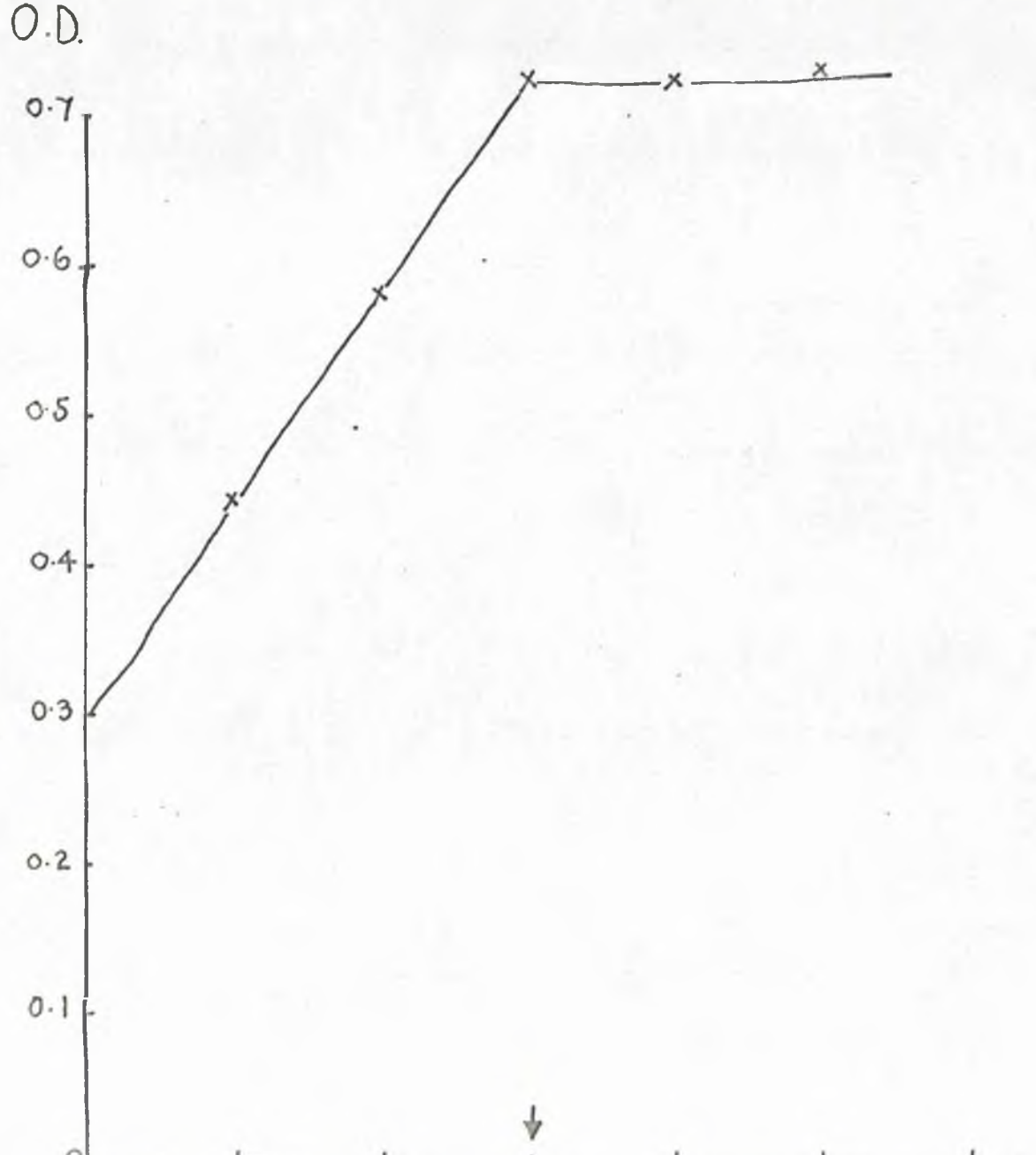


FIG. 2c.2 The standardization of PCMB by Glutathione

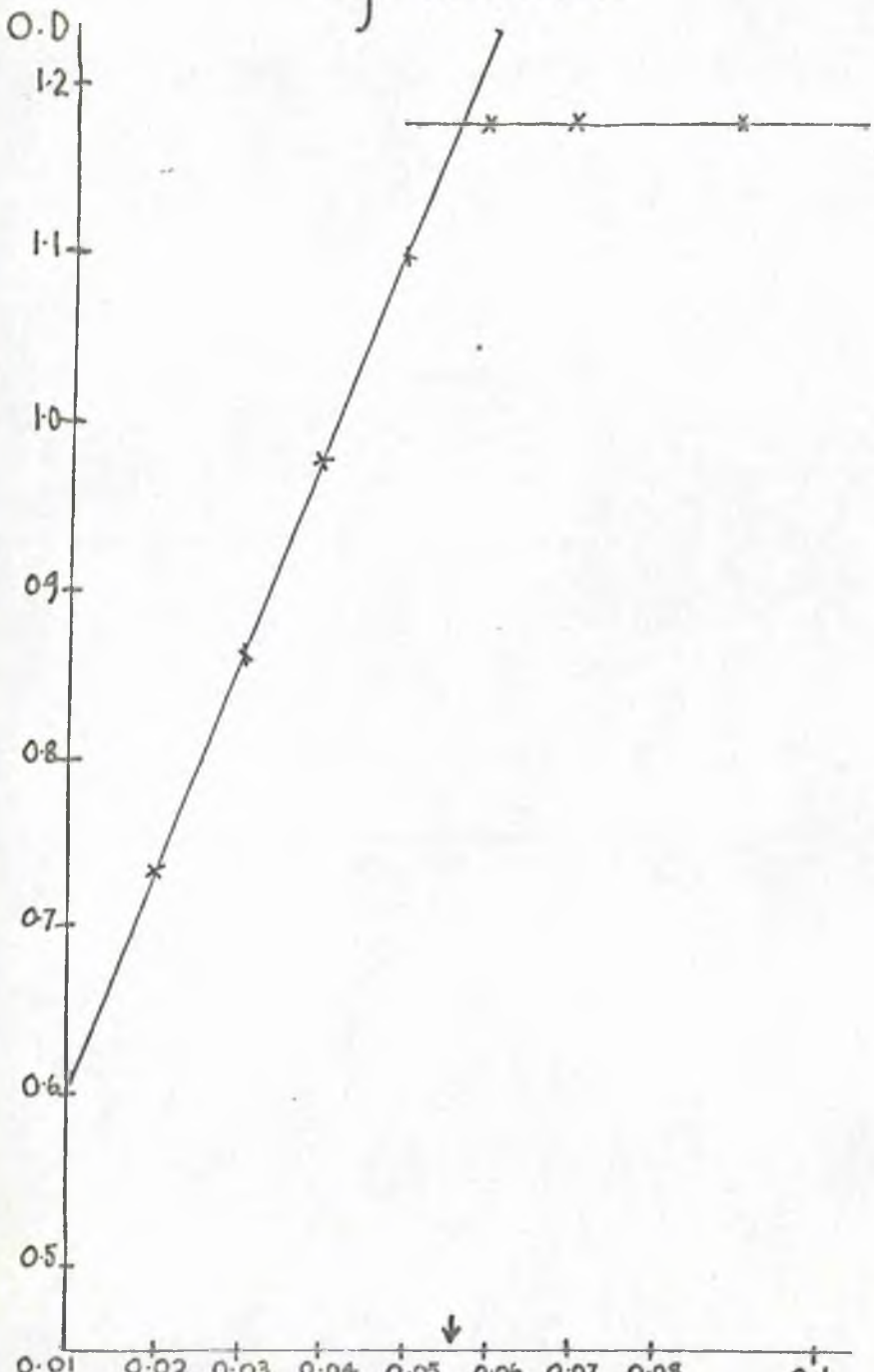


FIG. 2c.3 The Stability of Glutathione solution.

250 mμ.

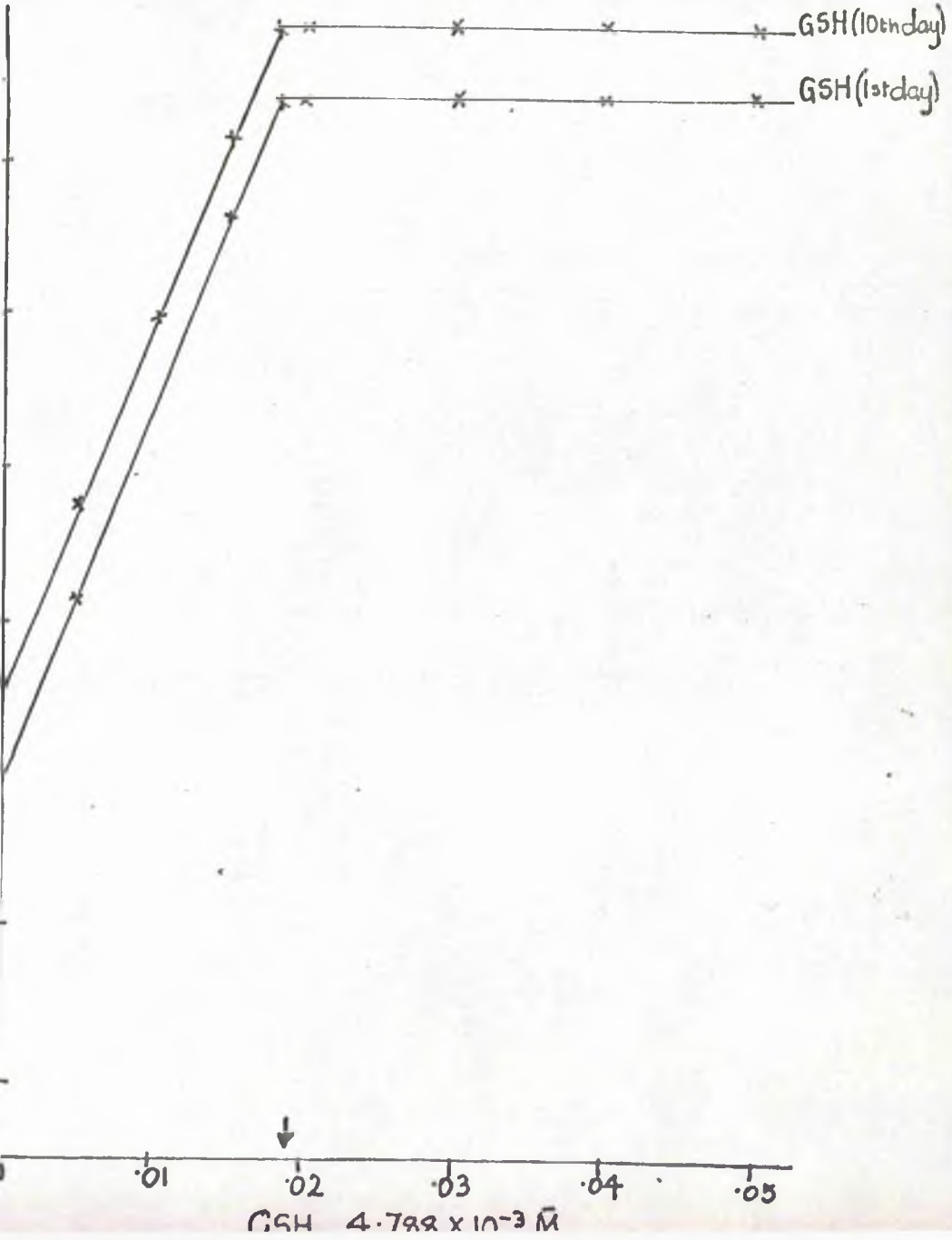


Fig. 2C.1. This graph and all subsequent ones were corrected for dilution before the end points in each case were determined. This is especially important when aliquot volumes of over 0.05 mls are added in PCMB titrations when the initial volume is only 3.0 mls.

Glutathione stability in standardisation procedures: Fig. 2C.3

Fresh glutathione standard was titrated with PCMB which was in turn retitrated with glutathione which had been stored at 1-2°C for 4 and 10 days.

The results show that glutathione solution (4.788×10^{-3} M) is very stable for at least 10 days while kept at 1-2°C.

The detection of - SH groups in Hb A. (Reactive)

This experiment was designed to show the specificity of PCMB for - SH groups - especially of Haemoglobin - in view of the fact of the degree of haemolysis which takes place during perfusion.

A molecular weight of 68,000* is used for Hb A (Tristram and Smith, 1963) in the following assay. A correction factor may be used but makes little difference to the final result.

The haemoglobin content of fresh blood was determined by the Cyanomethaemoglobin method.

---

* An amino acid composition of human haemoglobin using M.W. 64,500 is also tabulated in Appendix 2.

Determination of haemoglobin molarity:

Exactly 1.5 mls fresh blood (107% Hb)

↓

Centrifuged

R.B.C. → washed carefully 3 x with isotonic saline (0.9%)

Plasma pipetted → waste

↓

Centrifuged

Supernatant discarded

↓

Volume made up to 1.5 mls with 0.1 $\bar{M}$ Phosphate buffer and a further dilution of 3 mls distilled water i.e. Dilution x 3, was made.

A dilution of 300% was found to be necessary in order to estimate the reactive - SH groups. This then made the optimal haemoglobin Molarity about 8.0×10^{-4} $\bar{M}$ thus enabling adequate slitwidth adjustment of the blank.

1.5 mls of the original whole blood contains 0.238 g/Hb

= 107%

After dilution:

1 ml of the haemolysate = $\frac{0.238}{4.5}$ g/Hb.

= 0.0528 g/Hb.

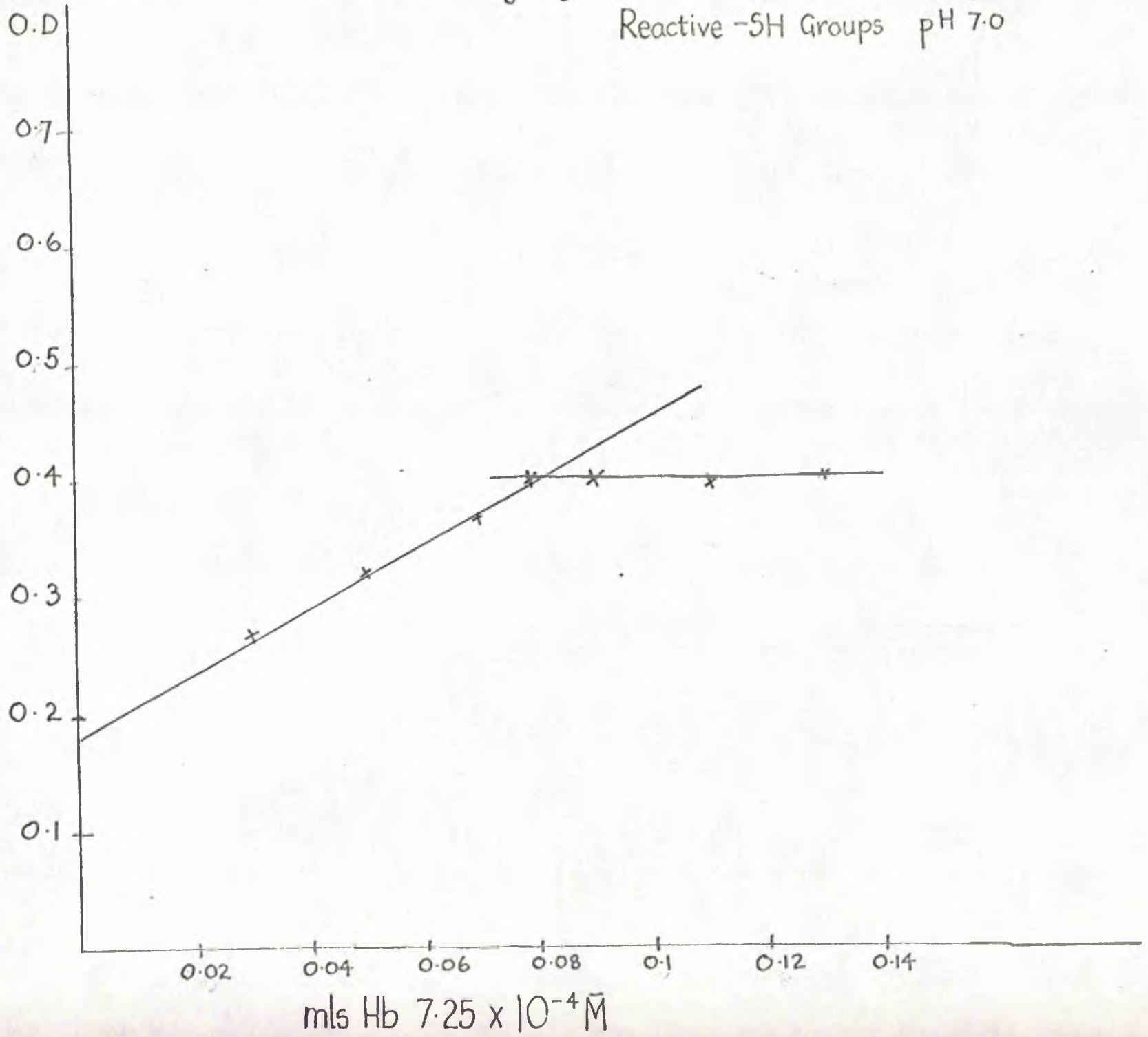
Thus the Molarity of the Hb solution

$$= \frac{52.8}{68,000} = 7.764 \times 10^{-4} \bar{M}$$

Titration of - SH content of Hb A (Reactive)

PCMB (pH 7.0) was standardised by GSH as above.

FIG. 2c·4 The Sulfhydryl content of Haemoglobin A. @ 250 mμ.



$= 4.925 \times 10^{-5}$ M

thus (Fig. 2C.4)

3 mls of 4.925×10^{-5} M PCMB = 0.079 mls Hb solution of x M

thus Hb solution = 1.87×10^{-3} in - SH.

But the Haemoglobin solution prepared above was 7.764×10^{-4} M

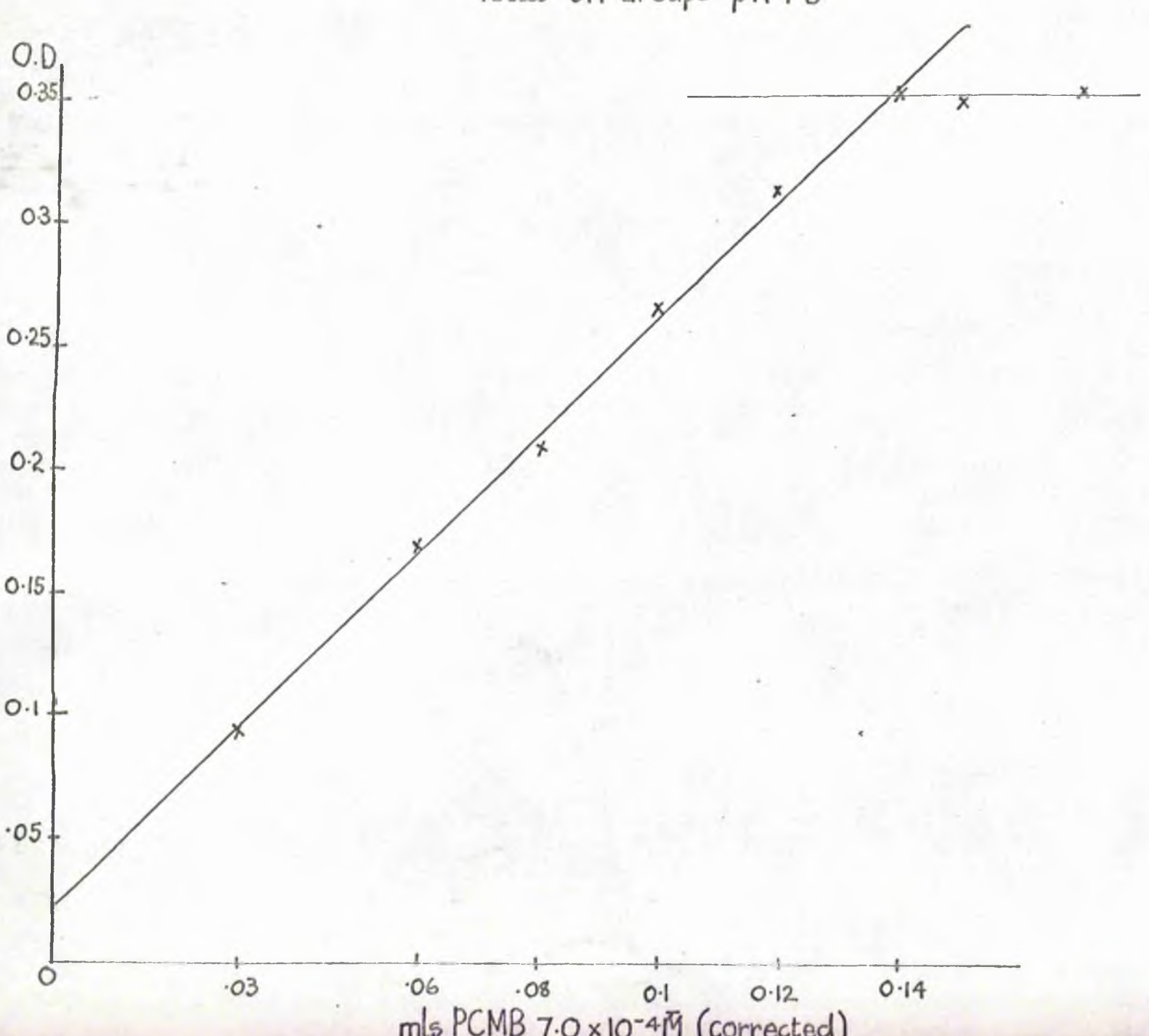
thus the number of - SH/mole

$$= \frac{1.87 \times 10^{-3}}{7.764 \times 10^{-4}}$$

$$= 2.406 \text{ - SH/mole/Hb.}$$

If a M.W. of HbA of 66,700 is used above then the - SH/mole Hb value = 2.37. So the exact M.W. of haemoglobin is not critical in this particular analysis.

FIG. 2c.5 The Sulfhydryl content of Haemoglobin A @ 255mμ



The total - SH groups of Hb A. Fig. 2C.5.

As mentioned earlier PCMB may be used to identify not only the readily available (reactive) sulfhydryl groups of HbA but may be used for the determination of total sulfhydryl groups.

The conditions of this assay are the following.

1) An acetate buffer at pH 4.6 is used.
2) A number of aliquots of the haemoglobin solution are left to react with varying concentrations of PCMB for at least 3 hours.
3) The absorption at 255 mμ is taken using the protein solution alone as the blank.
4) Each result is corrected by subtracting the respective PCMB absorption.

Varying volumes of 0.01-0.15 mls of standardised PCMB solution (7.0×10^{-4} M) were allowed to react with separate 3 ml samples of a standardised haemoglobin solution at pH 4.6 for 3 hours at room temperature.

Absorption was read at 255 mμ. The blanks consisted of 3 mls of Hb solution to which was added the corresponding volume 0.01 → 0.15 mls of distilled water.

The increase in absorption due to - SH binding was corrected by subtracting the absorption of the PCMB added (using buffer only as blank) to 3 mls buffer in 0.01 → 0.15 ml. increments.

From the interception of the two axes the End Point is apparent. The initial graph must be corrected for PCMB

absorption alone.

This experiment was repeated twice with similar results i.e. 6.08 and 5.97 moles of PCMB which are bound per mole of Hb A. The increase in detectable - SH groups from 2 to 3 moles/PCMB per mole of Hb A at pH 7.0 to 6 moles PCMB per mole of Hb A at pH 4.6, is undoubtedly due to the adverse effect of acidic conditions on the Haemoglobin molecule causing denaturation.

$$\text{PCMB Molarity in titration} = \frac{2 \text{ ml} \times 6.942 \times 10^{-6}}{0.12 \text{ ml}}$$

where 2 ml x 6.942×10^{-6} = volume and molarity of Hb solution and 0.12 ml = volume of PCMB at end point.

PCMB Molarity $\therefore$ = 115.2×10^{-6} M

The stock PCMB solution = 7.0×10^{-4} M

$$\therefore \text{ no. of - SH/mole Hb} = \frac{7.0 \times 10^{-4}}{1.152 \times 10^{-4}} = \underline{\underline{6.08}}$$

$\therefore$ Total no. of - SH groups in Hb A = 6.08/mole.

At this stage it is worthwhile to summarise the individual methods for the preparation of haemoglobin for the respective reactive only and total sulfhydryl estimations.

Reactive only - SH.
pH 7.0
Phosphate buffer
1.5 mls blood

↓

Centrifuged

↓ → plasma discarded

Cells washed carefully with 0.9% saline. 3-4 times.

↓

Centrifuged

↓ → Supernatant discarded

To the washed RBC's the volume is made up to 1.5 mls with 0.1 $\bar{M}$ phosphate buffer pH 7.0 and 3 mls of distilled water was added to give total volume of 4.5 mls.
Mixed well but gently.

↓

Centrifuged

↓ → ppt. discarded

Supernatant used for reactive - SH determination in Hb A.

As the Hb content of the original blood was known, the Molarity may be easily derived, e.g. 7.764×10^{-4} $\bar{M}$

0.01 ml increments of the solution was titrated against 3 mls soln A (PCMB) 4.925×10^{-5} $\bar{M}$

Total - SH
pH 4.6
Acetate buffer
1.5 mls blood

↓

Centrifuged

↓ ← plasma discarded

Cells washed carefully with 0.9% saline 3-4 times.

↓

Centrifuged

↓ ← Supernatant discarded

Volume (1.5 mls) reconstituted with 0.33 $\bar{M}$ Acetate buffer, pH 4.6, and 3 mls of distilled water was added to give total volume of 4.5 mls. Mixed well but gently

↓

Centrifuged

↓ ← ppt. discarded

Supernatant used in Assay.
1 ml taken and diluted to 100 mls with acetate buffer. Hb = 6.942×10^{-6} $\bar{M}$.
The stock PCMB solution 7.0×10^{-4} $\bar{M}$ was titrated in 0.01 ml increments against 2 mls of 6.942×10^{-6} $\bar{M}$. Hb solution - the latter having been diluted to 3 mls with 1 ml of water (dist).

FIG 2c.6. The sulfhydryl assay of Plasma.

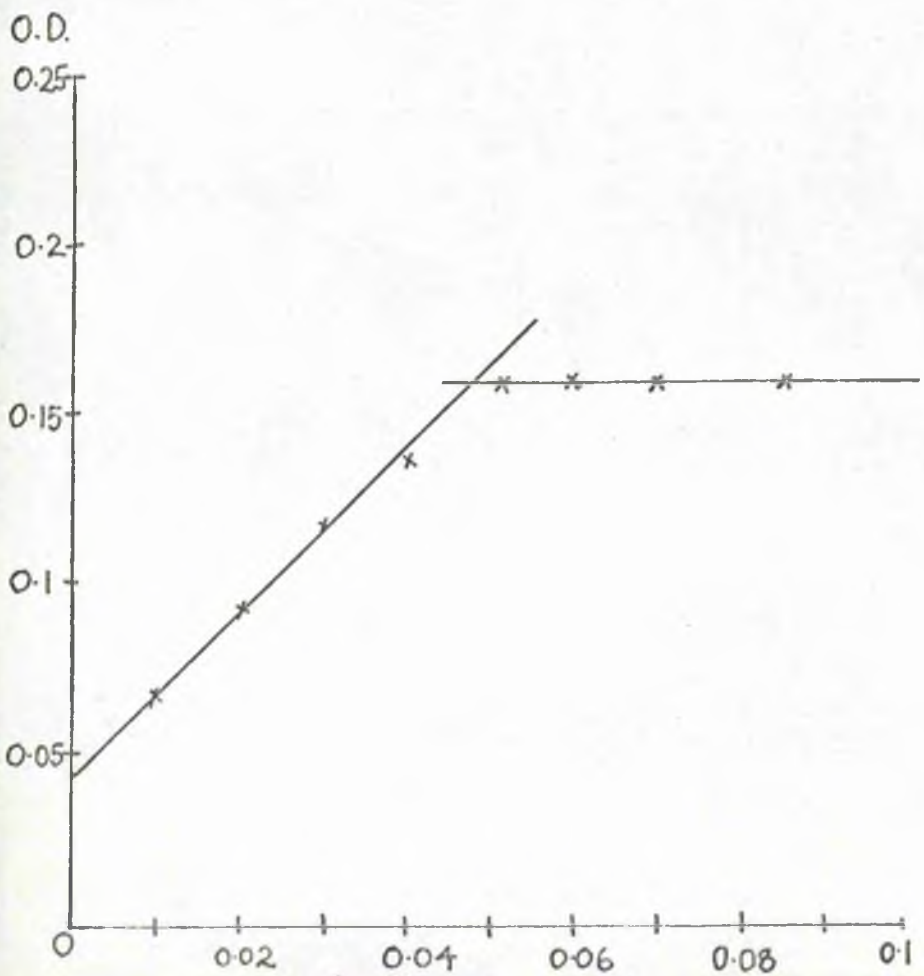
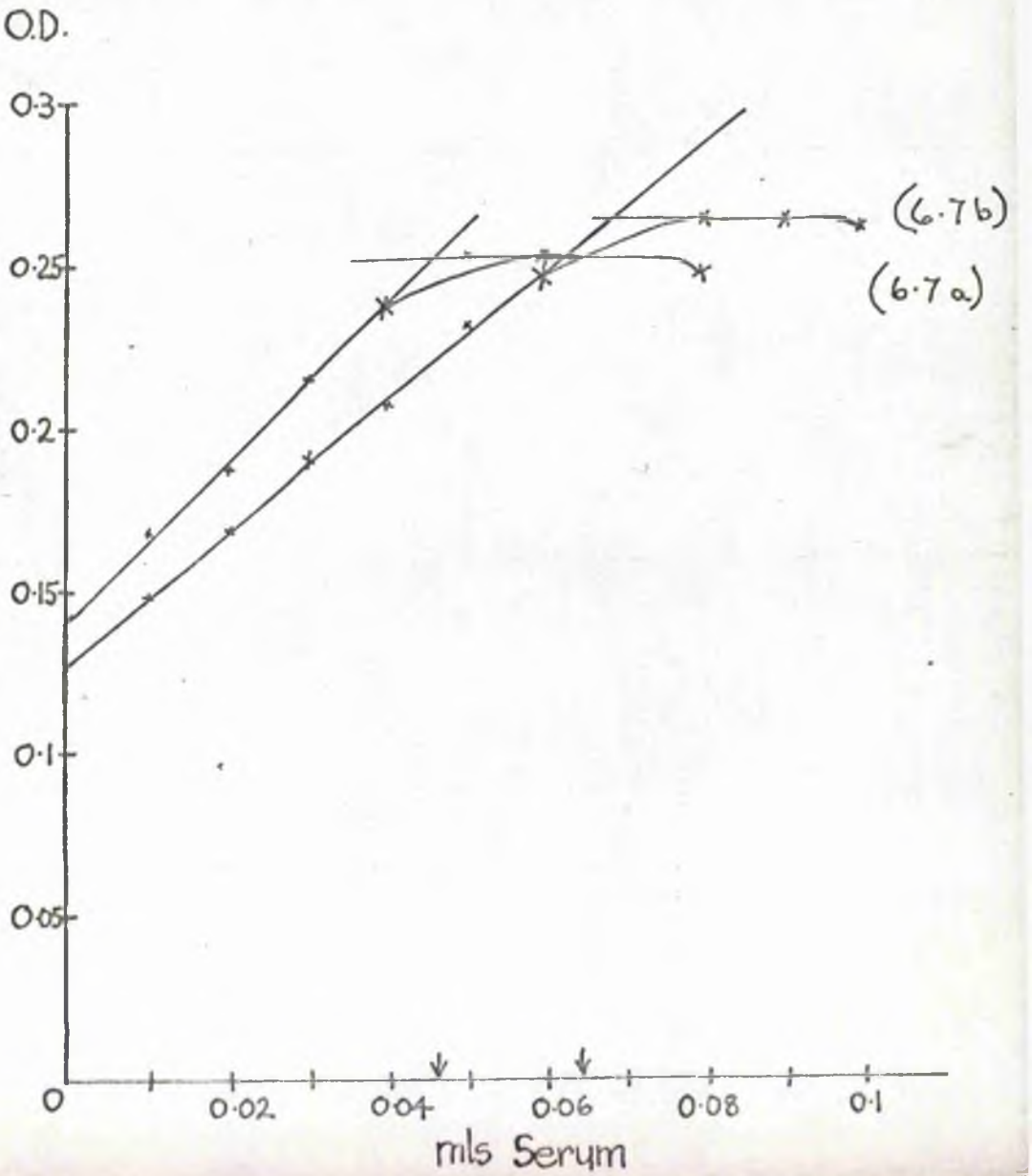


FIG 2c.7 The sulfhydryl assay of Plasma @ 250 mμ pH 7·0



The sulfhydryl assay of plasma. Fig. 2C.6

Since standard - SH titrations with PCMB gave such exacting end points, varying volumes of standard sulfhydryl (red. glutathione) concentrations were added to a specific volume of fresh plasma. Knowing the - SH content of the GSH added, the end point of the titration and the concentration of the standardised PCMB - the - SH content of plasma may be accurately determined.

3 mls of 1.721×10^{-5} M PCMB was titrated with plasma (1 ml containing 0.16 mls of 4.79×10^{-3} M GSH)

The end point on graph was 0.0515 mls.

Thus

$$\frac{3 \times 1.721 \times 10^{-5}\ \text{M}}{0.0515} = \text{M } [\text{SH}]$$

. . the total readily available - SH at end point (reactive - SH) = 1.002×10^{-3} M (GSH and plasma)

A = 3.306×10^{-2} gms/L/" - SH".

but the GSH is 4.79×10^{-3} M (from preliminary standard titration)

. . In 4.79×10^{-3} M GSH solution $\rightarrow$ 1.87×10^{-4} gms GSH in 0.16 mls.

B = 2.0×10^{-5} gms/SH in 0.16 m/GSH

from A there are 3.3066×10^{-2} gms/L/SH in plasma/GSH mixture.

and thus at end point (0.0515 mls) $\rightarrow$ 1.703×10^{-6} gms SH

C = 1.703×10^{-6} gms SH

2.0×10^{-5} gms SH in 0.16 mls GSH was added to 1 ml plasma of gms - SH. Total volume = 1.16 mls.

But only 0.0515 mls was needed for end point.

(0.16 ml is 13.8% of 1.16 mls)

∴ at end point 0.0515 mls x 0.138 = mls of GSH contributed to end point = 0.0071 mls.

∴ Volume of plasma at E.P. = 0.0515 -- 0.0071 = 0.0444 mls plasma.

from above: GSH present in the 1.16 mls (mixture) = 2.0×10^{-5} gms/SH.

∴ at E.P. $2.0 \times 10^{-5} \times 0.0444$ = 0.888×10^{-6} gms/SH (due to GSH)

∴ - SH in plasma at E.P.

= 1.703×10^{-6} gms/SH (from C)

- $\frac{0.888}{} \times 10^{-6}$

= 0.815×10^{-6} gms - SH in 0.0444 mls plasma.

∴ [SH] in plasma = 5.562×10^{-1} mM/SH/L.

It is worth mention at this point that the mean glutathione concentration in whole blood = 35.4 mgms/100 mls. (Geigy 5th Ed.). Mean range 26.9 - 41.4. None is normally present in plasma.

The results from fig. 2C.6 gives a slightly higher sulfhydryl concentration in plasma than other methods. This is undoubtedly due to its higher specificity to and reactivity with, these groupings. It agrees well with results from the mean amperometric titration method (0.53 mM/SH/L), Weissman et al., 1950.

Sulfhydryl assay of plasma - stored for 3 days at 2°C fig. 2C.7a.

Two assays were done which followed the same procedure as above.

GSH = 0.005 M̄

0.15 mls GSH was added to 0.85 ml plasma and mixed thoroughly. 0.01 ml increments of plasma were added to 3 mls 1.721×10^{-5} M̄ PCMB.

Absorption was measured at 250 mu after thorough mixing of each addition in silica cells.

End point (Fig. 2C.7a) = 0.046 mls.

B [SH] due to GSH in 0.15 mls = 0.246×10^{-4} gms/SH.

C In 0.046 mls (E.P.) there are

1.7032×10^{-6} gms - SH.

A E.P. Molarity = 1.122×10^{-3} M̄ SH in mixed sample.

from B, 2.46×10^{-5} gms SH in 0.15 mls GSH was added to 0.85 mls plasma of $\propto$ gms - SH.

At E.P. $0.15 \times 0.046 = 0.0069$ mls = volume of GSH contributed to E.P.

∴ Volume of plasma participating at E.P. =

$0.046 - 0.0069 = 0.0391$ mls plasma

[GSH] contributed at E.P.

$= 0.046 \times 2.46 \times 10^{-5}$ gms/SH $= 1.13 \times 10^{-6}$ gms/SH.

From titration. C

Total [SH] in 0.046 mls = 1.7032×10^{-6} gms - SH

∴ in 0.0391 mls plasma in titration it follows

$(1.7032 - 1.13)\ 10^{-6}$ gms/SH

$= 0.5732 \times 10^{-6}$ gms/SH @ E.P.

$\therefore$ [SH] in gms/L in plasma (original)

$$= \frac{5.732 \times 10^{-7} \times 1000}{0.0391}$$

$= 4.446 \times 10^{-1}$ mM/SH/L

Fig. 2C.7(b) With the same sample of plasma a different volume of glutathione standard was added.

0.08 mls of 5.0×10^{-3} M GSH was added to 0.92 mls plasma with gentle but thorough mixing.

PCMB = 1.721×10^{-5} M.

0.01 ml increments of plasma/GSH mixture was added to 3 mls 1.721×10^{-5} M PCMB and absorption measured at 250 mμ after each addition and thorough mixing.

E.P. = 0.064 mls.

A E.P. molarity = 8.067×10^{-4} M - SH in plasma/GSH mixture

B - SH due to GSH in 0.08 mls = 1.316×10^{-5} gms - SH.

C At E.P. - 1.7×10^{-6} gms - SH (TOTAL)

from B 1.316×10^{-5} gms - SH in 0.08 mls GSH added to 0.92 mls plasma of gms - SH. Total volume = 1 ml

But only 0.064 mls needed for end point

$\therefore$ Volume GSH contributing to E.P. (0.064 mls)

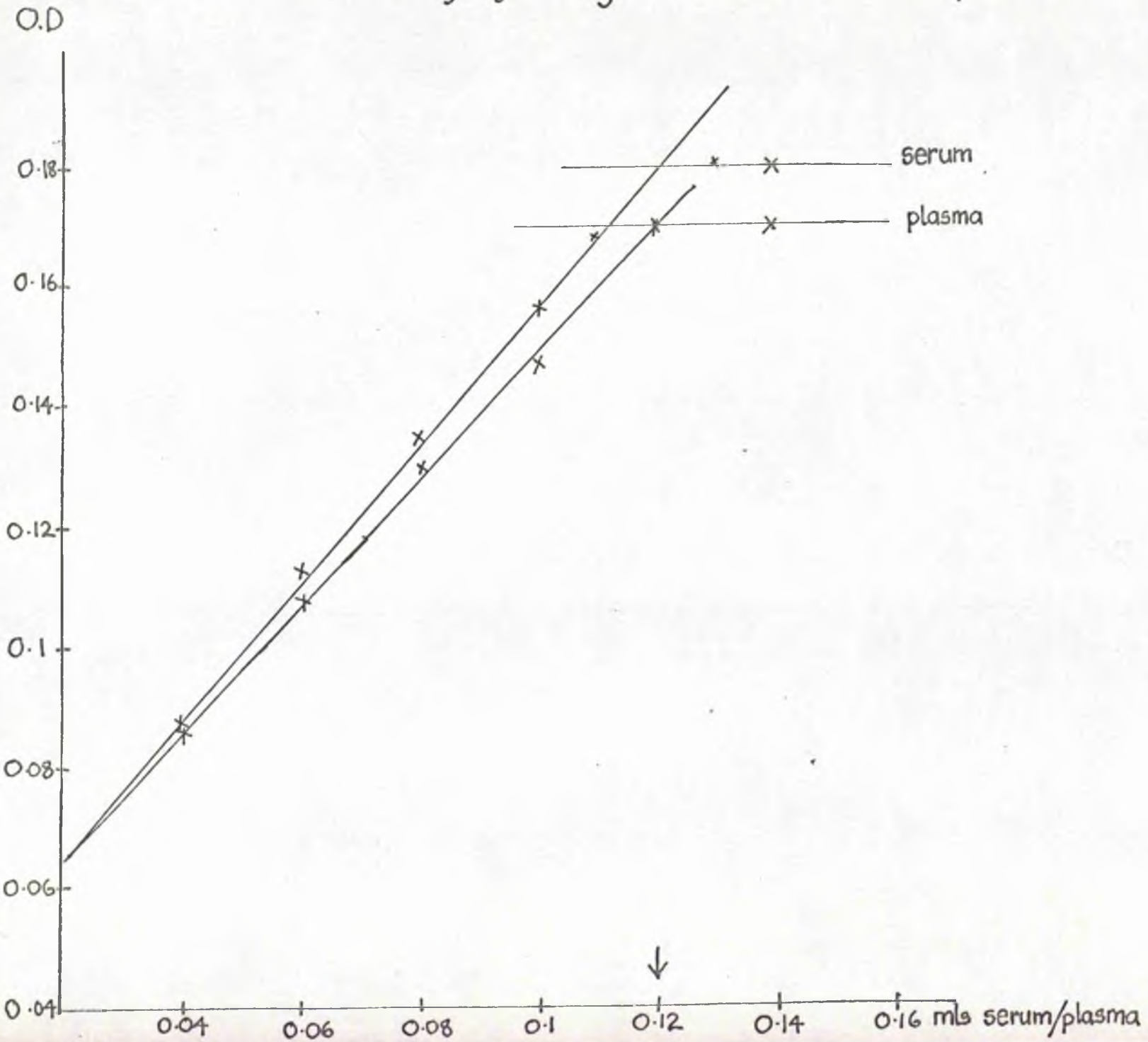
= 0.00512 mls.

[GSH] contributing to E.P. = 8.42×10^{-7} gms - SH.

Volume of plasma in sample at E.P. = 0.064 - 0.00512

= 0.0589 mls plasma

FIG. 2c·8 The sulfhydryl analysis of fresh serum and plasma.



from C

$$\text{Total } [\bar{} \text{ SH}] \text{ (gms)} = (1.7 \times 10^{-6}) - (0.842 \times 10^{-6})$$

$$= 0.858 \times 10^{-6} \text{ gms - SH.}$$

∴ In 0.589 mls plasma at E.P. there are 0.858×10^{-6} gms - SH

$$= 4.415 \times 10^{-4} \text{ M/SH/L}$$

$$= 4.415 \times 10^{-1} \text{ mM/SH/L.}$$

Sulfhydryl analysis of fresh serum and plasma Fig. 2C.8.

Fig. 2C.8 shows the virtually identical results of - SH content using serum and plasma (fasting).

The end point in each case is 0.12 mls. Plasma or serum as titrant.

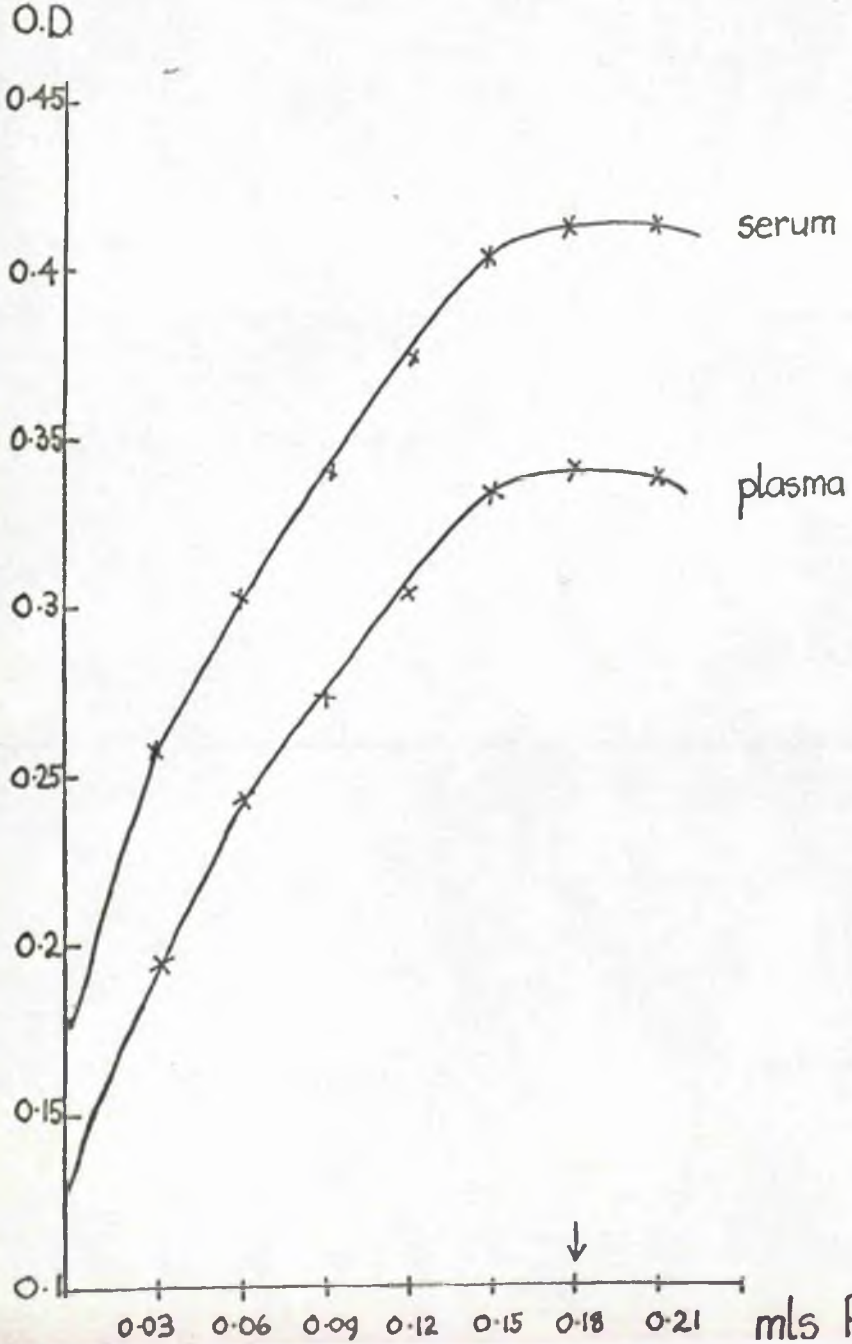
PCMB (1.721×10^{-5} M̄) was standardised by 2.4×10^{-3} M̄ GSH.

Both the plasma and serum give 4.302×10^{-1} mM/SH/L. That the sulfhydryl determination in both fluids are the same, suggests that fibrinogen, prothrombin, enzymes and other proteins of plasma concerned in the blood clotting mechanism do not have any significant free detectable (reactive) sulfhydryl groups in non denatured sample.

A different sample of fresh serum and plasma was titrated with a more concentrated PCMB solution. Identical results were again obtained using serum and plasma from the same fasting subject, see Fig. 2C.9.

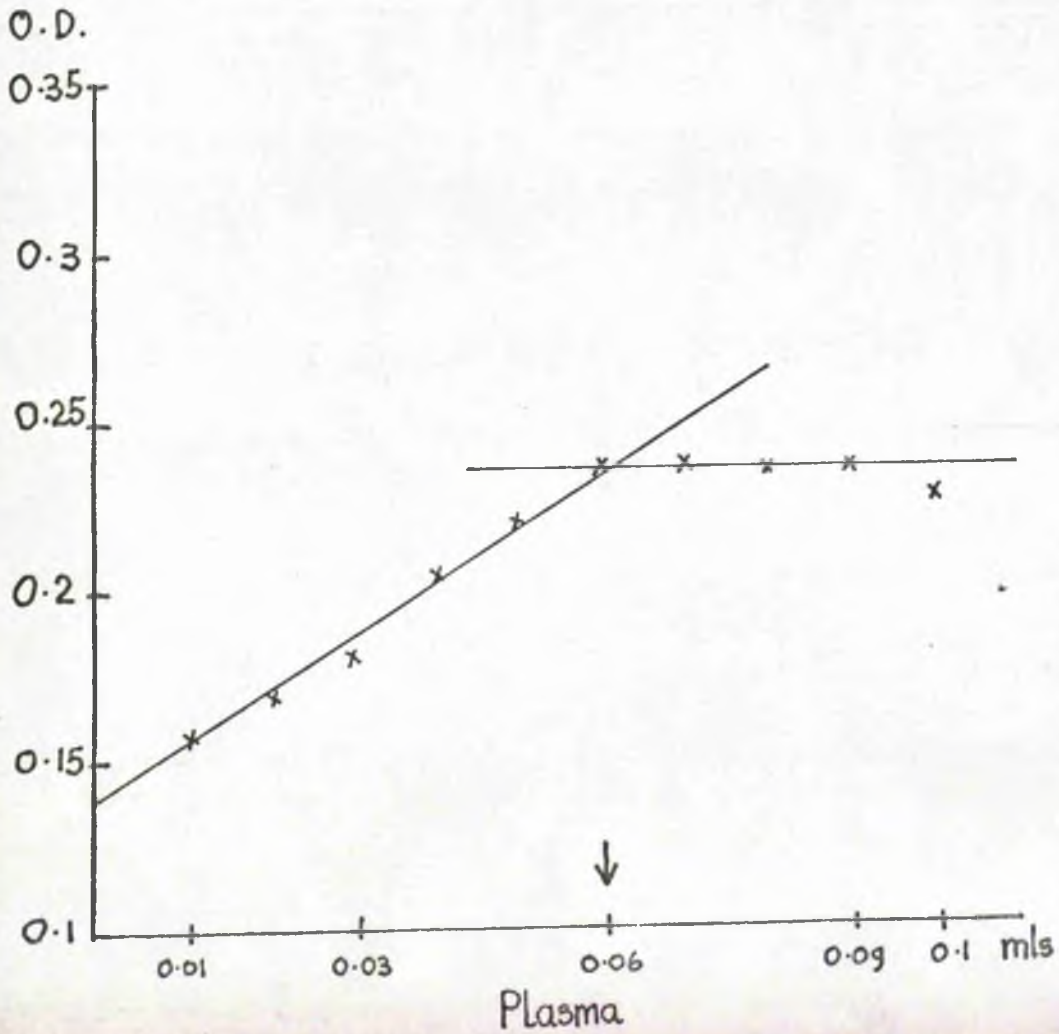
1 ml of stock PCMB solution was diluted to 25 mls and standardised. PCMB Molarity = 3.16×10^{-5} M̄.

FIG. 2c.9 The sulfhydryl analysis of fresh serum



and plasma.

FIG. 2c·10 Denaturation of plasma by heat.



The end point for both serum and plasma seen in Fig. 2C.9 is 0.18 mls.

The sulfhydryl content of this serum and plasma is thus

$$52.66 \times 10^{-5}\ \bar{M}$$

$$= 0.5266\ \text{mM/SH/L.}$$

<u>The effect of heat on plasma:</u> <u>Denaturation by physical means.</u> Fig. 2C.10.

Plasma was subjected to 55°C for 45 minutes and taken up to 65°C for 5 minutes terminally - in a thermostatically controlled water bath.

There was a large increase in detectable - SH groups, three times the value found in normal fresh plasma.

Denatured plasma (by heat) = 1.58 mM/SH/L.

Fresh plasma - Mean - SH value = 5.2×10^{-1} mM/SH/L.

4 day old plasma = 6.32×10^{-1} mM/SH/L.

<u>Partial denaturation by heat.</u> Fig. 2C.11

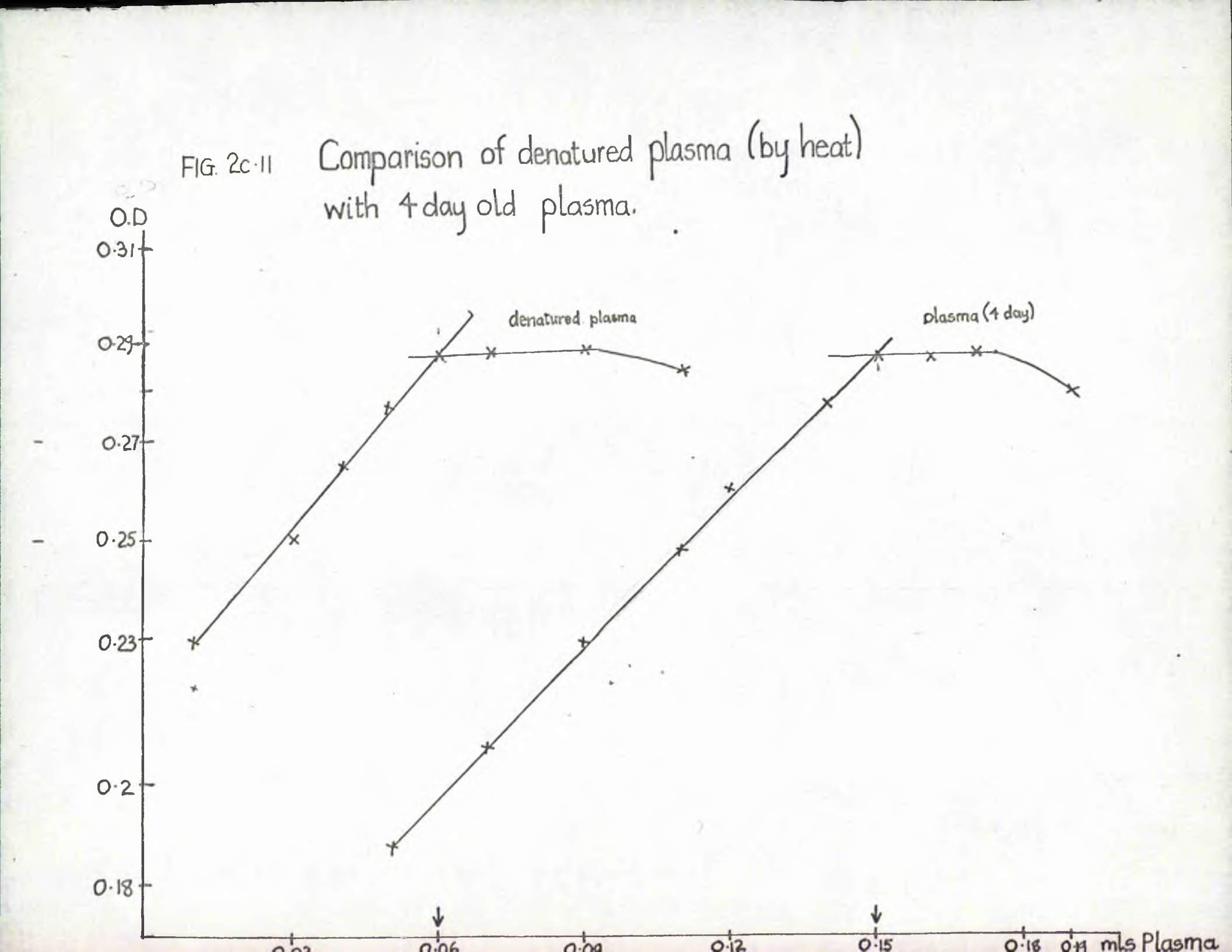
This experiment was repeated twice with 4 day old plasma. Here it was subjected to 45°C immediately and the temperature increased to 65°C over 5 minutes.

Three interesting results were obtained here:

1) The end point of one titration was identical with the above titration, the - SH concentration being 1.6 mM/SH/L.

2) In a similar titration the end point came later at 0.08 mls, the - SH content of the plasma being at this point

FIG. 2c·11 Comparison of denatured plasma (by heat) with 4 day old plasma.

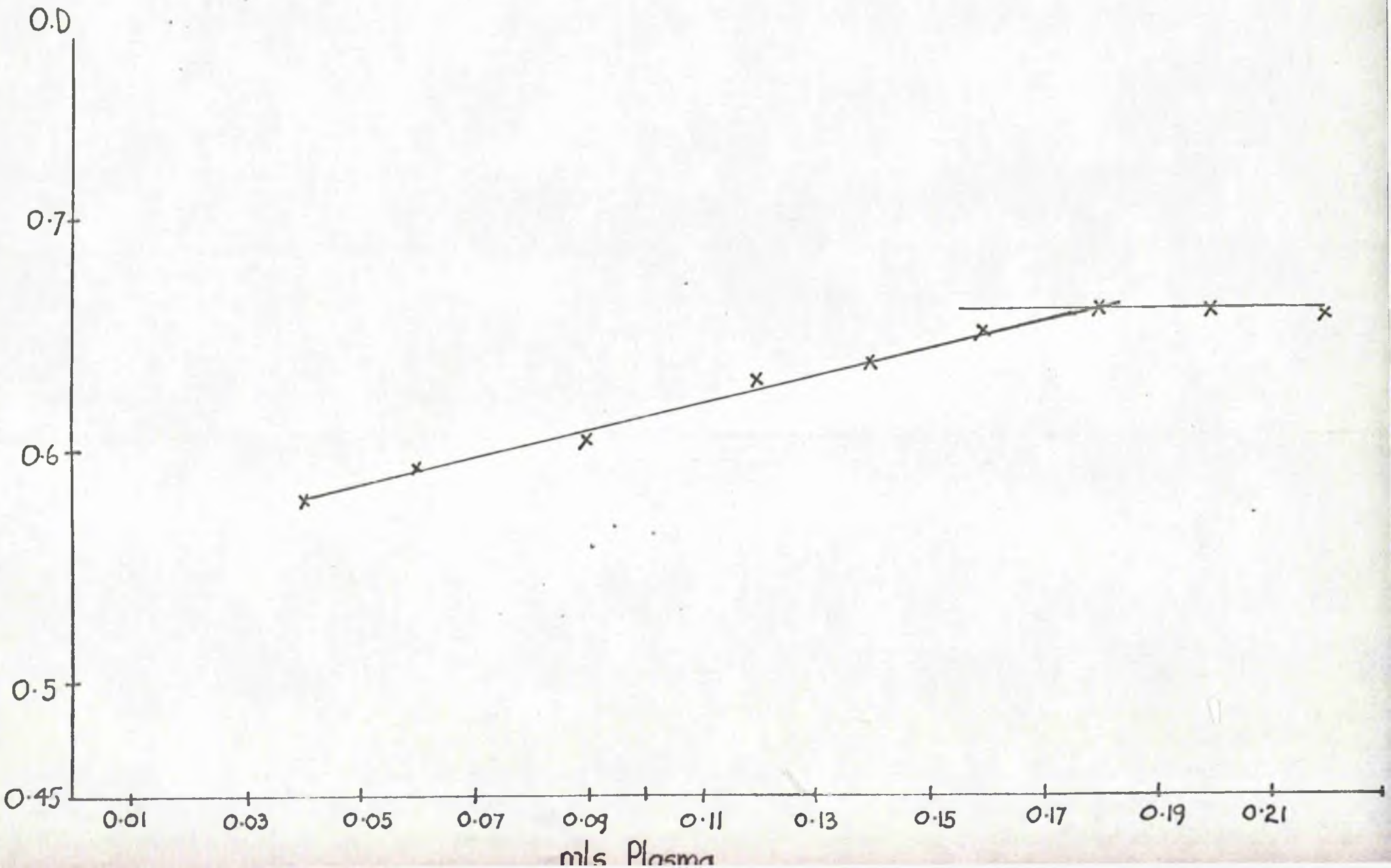


1.18×10^{-3} M. This is interesting because both plasmas were subjected to exactly the same conditions and yet in the latter case there is only a 50% increase in sulfhydryl reactivity with PCMB as compared with control and denatured plasma (heat) results of 2C.10 and the first part of 2C.11.

This suggests that after partial denaturation has taken place (by this particular method) only a relatively small extra denaturing factor is necessary in order for complete denaturation (assumption) to be obtained. It is assumed that the figures arrived at in the earlier part of this experiment, 1.58 and 1.6, does possibly represent full denaturation since very similar results were obtained in the DTNB method using acetone as denaturing agent.

3) The third point of interest is the fact that the rate of denaturation tends to be sigmoidal in character in that 80% or so of denaturation occurs early on in the denaturing process.

FIG. 2c.12 The effect of 8M Urea on Plasma – SH groups.



The effect of 8 M̄ urea on plasma: denaturation by chemical means. Fig. 2C.12.

Fresh plasma was titrated with PCMB in the presence of 8 M̄ urea (plasma/urea 66:33).

PCMB concentration = 8.93×10^{-5} M̄ (i.e. 3 25 stock).

As seen in fig. 2C.12 the intercepts occur at 0.18 mls plasma = 1.48 mM/SH/L.

This value is only 10% below the values found by heat denaturation. Equilibration time of the plasma/urea mixture was 15-30 minutes. The experiment shows that 90% plasma appears easily and efficiently denatured by a high concentration of urea.

The intercept is rather oblique due to a higher PCMB concentration (3 mls of stock solution A diluted to 25 mls with phosphate buffer) being used. More acute intercepts may be obtained with PCMB used in 1 → 25 dilution.

- Sulfhydryl determination of plasma with glutathione (reduced)

Glutathione freshly standardised was added in equal volumes with plasma to PCMB. The end point obtained as seen in Fig. 2C.13 was compared with

1) GSH alone and

2) Plasma alone.

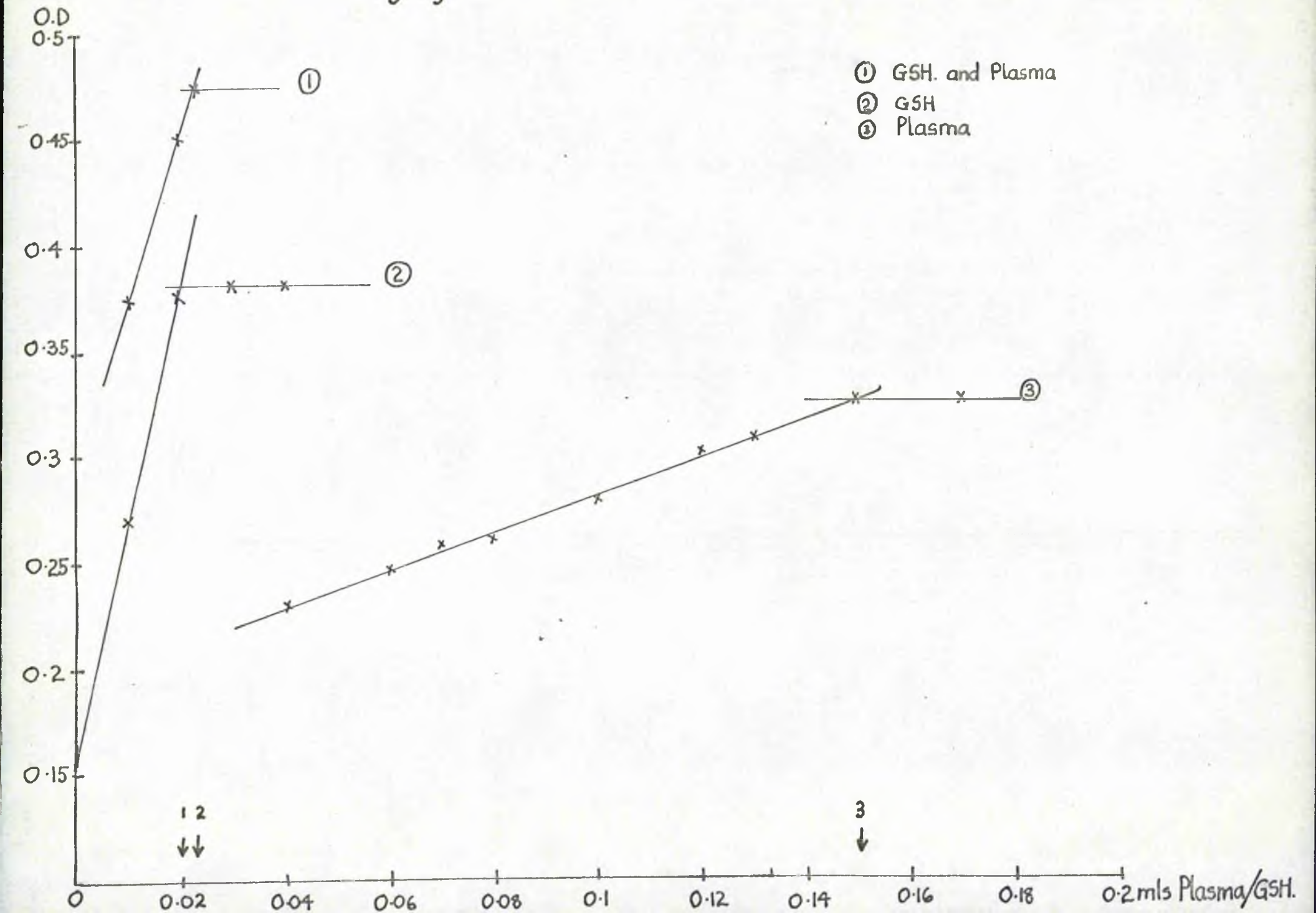
1) Glutathione Molarity = 4.8×10^{-3} M̄

End Point = 0.02 ml (GSH).

2) Plasma Molarity = 6.4×10^{-1} mM

End Point = 0.15 ml (Plasma).

FIG. 2c. 13. Sulfhydryl determination of Plasma with added Glutathione.



3) Mixed GSH/Plasma titration: 0.01 ml each of GSH and plasma were added to the 3 mls of standard PCMB solution, carefully but thoroughly mixed and immediately read at 250 mμ. The end point obtained was 0.023 mls GSH/Plasma mixture.

Since equal volumes of GSH and plasma were used to obtain the final intercept it follows that this point is dependent solely on the ratio of the concentrations of these - sulfhydryl donors.

The Molarity of Plasma and Glutathione are known - as above.

The percentage which plasma contributes to the End Point is thus $\frac{0.64}{4.8} \times 100 = 13.3\%$.

Thus it would be expected that glutathione would contribute approx. $\underline{\underline{86.7\%}}$ to the intercept.

The GSH/Plasma intercept = 0.023 mls.

$\therefore$ The mixed - SH Molarity = $\frac{9.6 \times 10^{-5}}{0.023} = 4.19 \times 10^{-3}$ M

$\therefore$ percentage contributed by GSH to end point

$$= \frac{4.19}{4.8} = 0.87.3$$

$$= \underline{\underline{87.3\%}}$$

Thus adding equal volumes of standard glutathione and plasma (known or unknown sulfhydryl concentration) to standardised PCMB solution enables a very accurate end point to be obtained.

The titration plots are more accurate and the intercept more distinct.

DISCUSSION.

PCMB has shown its high specificity and reliability in the detection of - SH groups. Due to its continual restandardisation by titration and that individual titrations have to be done on each aliquot assay - it is a very time consuming procedur

In the foregoing individual experiments however it has given interesting results.

1) In every mole/Hb there are at least 2 - SH groups which are readily available (reactive). This rises to at least 6 when haemoglobin is (a) either denatured or (b) a lower buffer pH is employed, which increases PCMB's reactivity of its ionised carboxyl group and hydroxyl ion affinity, to assay the total - SH.

Thus it is clear that not only has native haemoglobin present in fresh blood, many reactive - SH groups available for reaction (with PCMB), but that this factor has the capacity to increase some 300% if for any reason haemoglobin becomes denatured.

It therefore would appear most feasible that in open cardiac surgery oxygenation of blood and the trauma inflicted upon erythrocytes by the pump roller contributes in no small way to the increase in - SH content seen in the NEM and DTNB sections.

Haemoglobin when denatured fully loses its colour and no longer allows oxygen to associate. This is of rather theoretical value since the - SH results of blood and

plasma show no tendency towards this extreme situation whatsoever. However the fact is pertinent to this present investigation.

2) The use of added glutathione in the - SH assay of plasma is of great value in elucidating a more exact end point.

3) The good agreement of PCMB results of plasma - SH with published amperometric (polarographic) results is shown, e.g. 5.0-5.5 x 10^{-1} mM/SH/L.

Using the NEM method for - SH assay it will be remembered the mean value of 1.24 x 10^{-1} mM/SH/L was obtained. These figures indicate that PCMB has greater specificity for, or reactivity with - SH groups than NEM, and that the values found probably truly indicate the maximum - SH groups available since the amperometric method gives the same values and is a highly specific method in its own right as well.

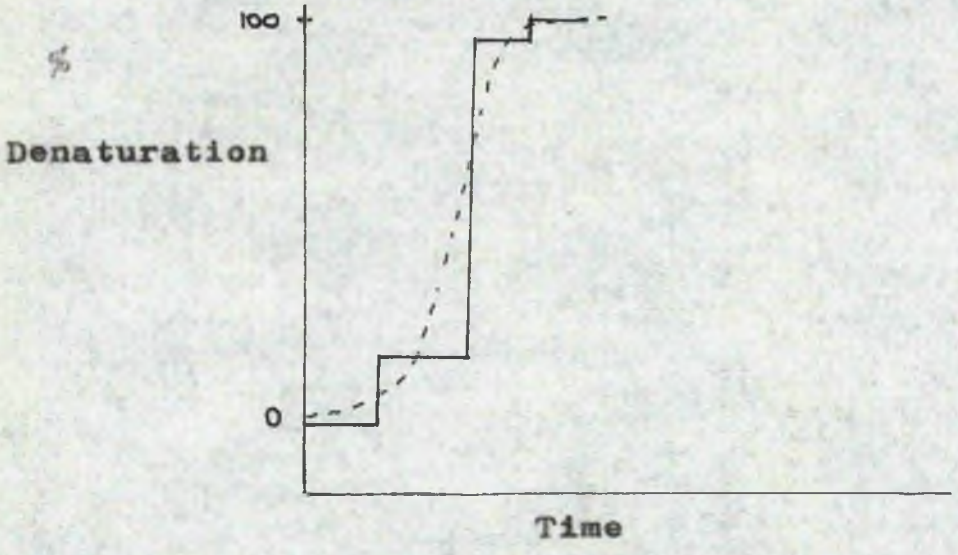
4) The - SH assay of plasma shows a decrease in - SH content with storage as shown in section 2B.

5) It is very interesting and important to find that no detectable difference was shown between the - SH content of fresh plasma and that of serum. Thus suggesting that - SH groups of the blood clotting proteins are not reactive in the native state.

6) Denaturation of plasma proteins by heat gave a large increase in detectable - SH groups. The comparison of

the long and short term heat treatment does suggest that plasma denaturation may well be a stepwise process rather than an all or nothing reaction. Steven and Tristram, 1958 suggest the former possibility in their work on "the reactivity of free amino groups in native and denatured ovalbumin towards fluorodinitrobenzene", although Mirsky and Anson, in 1935 proposed the all or nothing reaction in the denaturation of albumin.

The apparent sigmoidal character of the denaturing response has been mentioned, and thus the mechanism of plasma protein denaturation could be graphically represented thus:-



7) The results of the effect of 8 M̄ urea on plasma are of interest since they are in opposition with findings in the DTNB section in which urea appeared to have little effect on plasma denaturation as judged by virtually no increase in - SH groups. Therefore the use of differe

sulfhydryl reagents is of great importance in the qualitative and quantitative changes in - SH content of protein molecules.

CONCLUSION.

1) Owing to the necessity of frequent standardisations the use of PCMB has limitations in routine - SH analysis.
2) The number of - SH groups in native and donatured haemoglobin has been demonstrated and there appears to be an approximate 300% increase in - SH from the native to the denatured state.
3) The advantage of adding known concentrations of glutathione to plasma of unknown - SH content has been demonstrated.
4) Good agreement has been obtained between the PCMB - SH assay of plasma and published amperometric results, i.e.

$$5.0 - 5.5 \times 10^{-1} \text{ mM/SH/L.}$$

5) A decrease in - SH content with storage is again demonstrated.
6) The blood clotting proteins do not appear to have such reactive - SH groups as haemoglobin, albumin, the globulins and probably plasma enzymes.
7) Denaturation of plasma by heat yields a 300% increase in - SH groups.
8) A similar increase in - SH groups was found when plasma was denatured by a high concentration of urea.
9) A graphical representation has been made of denaturation based on the above data.

SECTION 2 D.

INTRODUCTION

Bis p. nitro phenyl disulphide. (PNPD): Section 2D.

This fourth sulphydryl reagent was used very successfull on both blood and plasma.

p. nitrophenyl disulphide reacts quantitatively with many marcaptans to liberate one mole of p. nitrophenyl mercaptan (p. nitrobenzene thiol) per mole of mercaptan. The reaction takes place at pH 8.0 and as the p. nitrophenylmercaptan anion is highly coloured (ϵ_m = 13,600 at 412 mμ) it can be used as a measure of thiol concentration.

Its disadvantages are:

1) The disulphide is very insoluble in water
2) It shows slow reactivity with some sulfhydryl groups e.g. cysteine and β mercaptoethylamine, but is more reactive with other compounds like β mercaptoethanol and α toluenethiol.

It is of interest that DTNB is the water soluble derivative of PNPD.

Because of the insolubility of PNPD in water, acetone is used as solvent - and therefore the sulfhydryl groups estimated by this method are the total number in the specimen rather than just the reactive groups because of the denaturing effect of the solvent. This appears to be the sole disadvantage of this method, other than the precarious nature of keeping a standard in a volatile solvent.

METHODS.

To test the validity of the release of one mole of p. nitrophenyl anion per mole of thiol the following tests were performed.

Reagents 30.8 mgm bis p. nitrophenyldisulfide dissolved in 100 mls Acetone and tightly stoppered.

Buffer 0.1 $\bar{M}$ pH 8.0.

4 mls 1 x 10^{-4} $\bar{M}$ glutathione in 4 mls acetone was mixed with 1 ml PNPD 1 x 10^{-4} $\bar{M}$ and

1 ml Phosphate Buffer.

The absorption was measured at once against a blank consisting of

4 mls water

1 ml Buffer

4 mls Acetone

1 ml PNPD 1 x 10^{-4} $\bar{M}$

the absorption of 412 mμ was 0.1375 (Mean of 3)

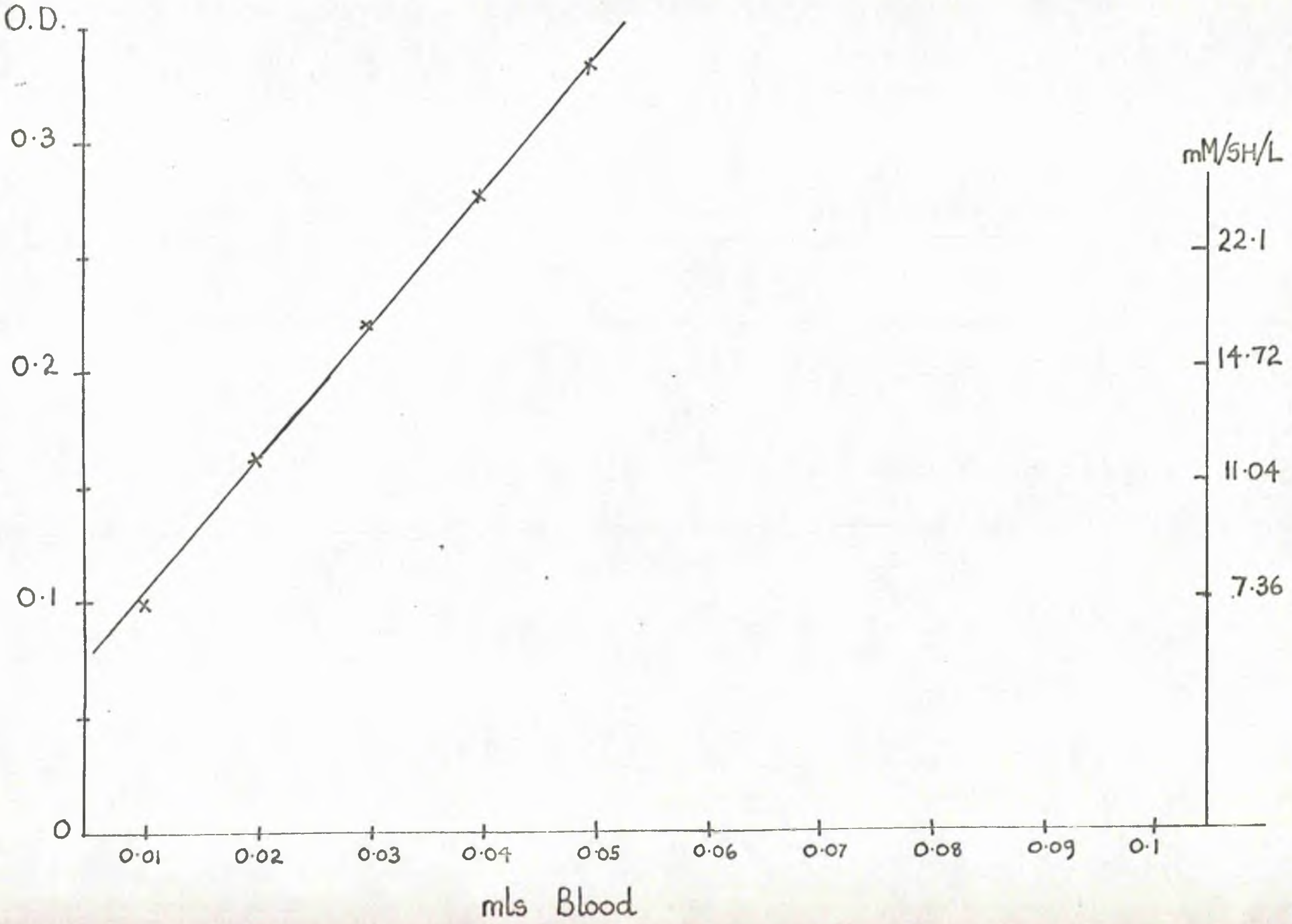
4 mls p. nitrophenyl mercaptan was diluted to 10 mls by 2 mls buffer and 4 mls of acetone.

The immediate absorption at 412 mμ was 0.1369 (Mean of 3

thus 4 mls 1 x 10^{-4} $\bar{M}$ GSH = 4 mls 1 x 10^{-4} PNPM

1 mole of - SH = 1 mole PNPM

FIG. 2D·1 The relation between blood mercaptans and liberated p·nitrophenyl anions.



Sulfhydryl concentration in Blood.

0.01 mls Blood were mixed well with

5 mls water and added to

2 mls buffer and

3 mls Acetone (Blank).

In the test solution 2.7 mls Acetone and 0.3 mls standard PNPD were used instead of 3 mls Acetone.

The calibration curve is shown in Fig. 2D.1. As can be seen, there is a perfect linear relationship between blood volumes 0.01 - 0.05 mls and increased P.N.P. anion.

5 blood specimens were sampled and the mean taken.

The results are about half value of these described by Ellman who shows values of 13.5 - 15.0 mM/SH/L. for males and 10.5 - 12.0 mM/SH/L for females.

In this case the mean value was 7.5 mM/SH/L.

Using a random 0.01 mls blood (female)

e.g. Mean O.D. @ 412 mμ = 0.105

$\therefore$ C in mM/SH/L = $\frac{0.105}{13.6} \times 1000 = 73.6$ A

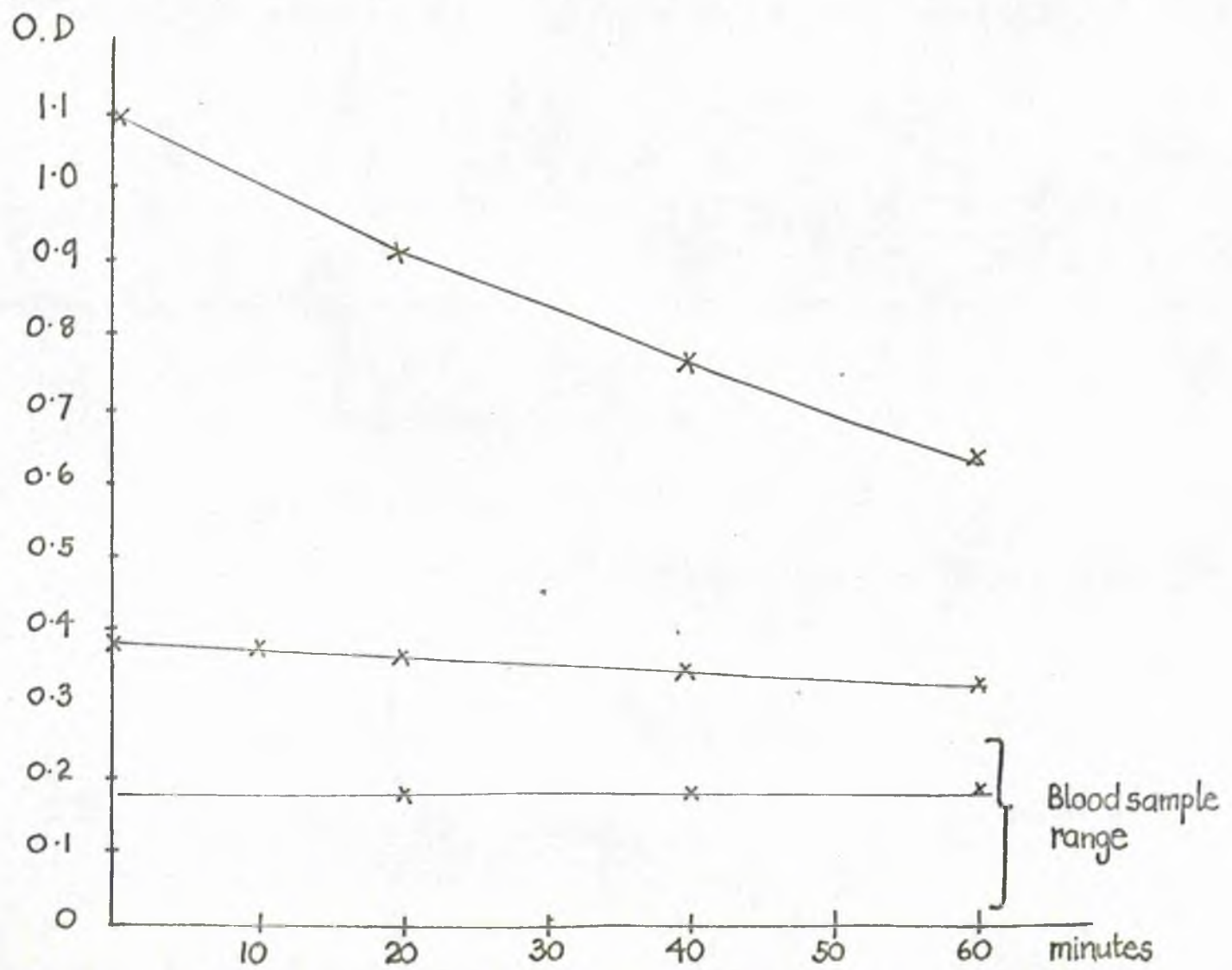
$= 73.6 \times 0.105$

$= \underline{7.5}$ mM/SH/L.

This method appears to be more accurate using 0.02 mls - 0.03 mls blood, for results agreeing with Ellman's were found, e.g. 12.66 mM/SH/L. (O.D. 0.174) in other female blood samples.

If the reactants are left for 60 mins. there is a minimal increase over the 3-5 min. results as follows:

FIG. 2D.2 The stability of varying concentrations of p.nitrophenyl anions with time.



	O.D.
2 mins	0.172
60 mins	0.174
90 mins	0.175

If the p. nitrophenyl anion liberated is of higher concentration than that found in this particular sulphydryl assay of blood, there is in fact a decrease in absorption over the 1 hr. period as seen in Fig. 2D.2. This is virtually negligible for blood volumes of 0.01-0.03 mls.

Results of blood samples Female blood samples.

O.D.	mM/SH/L.
0.174	12.66
0.17	12.6
0.154	11.33
0.16	11.77

Mean = 12.09 mM/SH/L.

Sulfhydryl concentration of plasma

In this assay 0.1 mls plasma was used and mixed with

1 ml Buffer

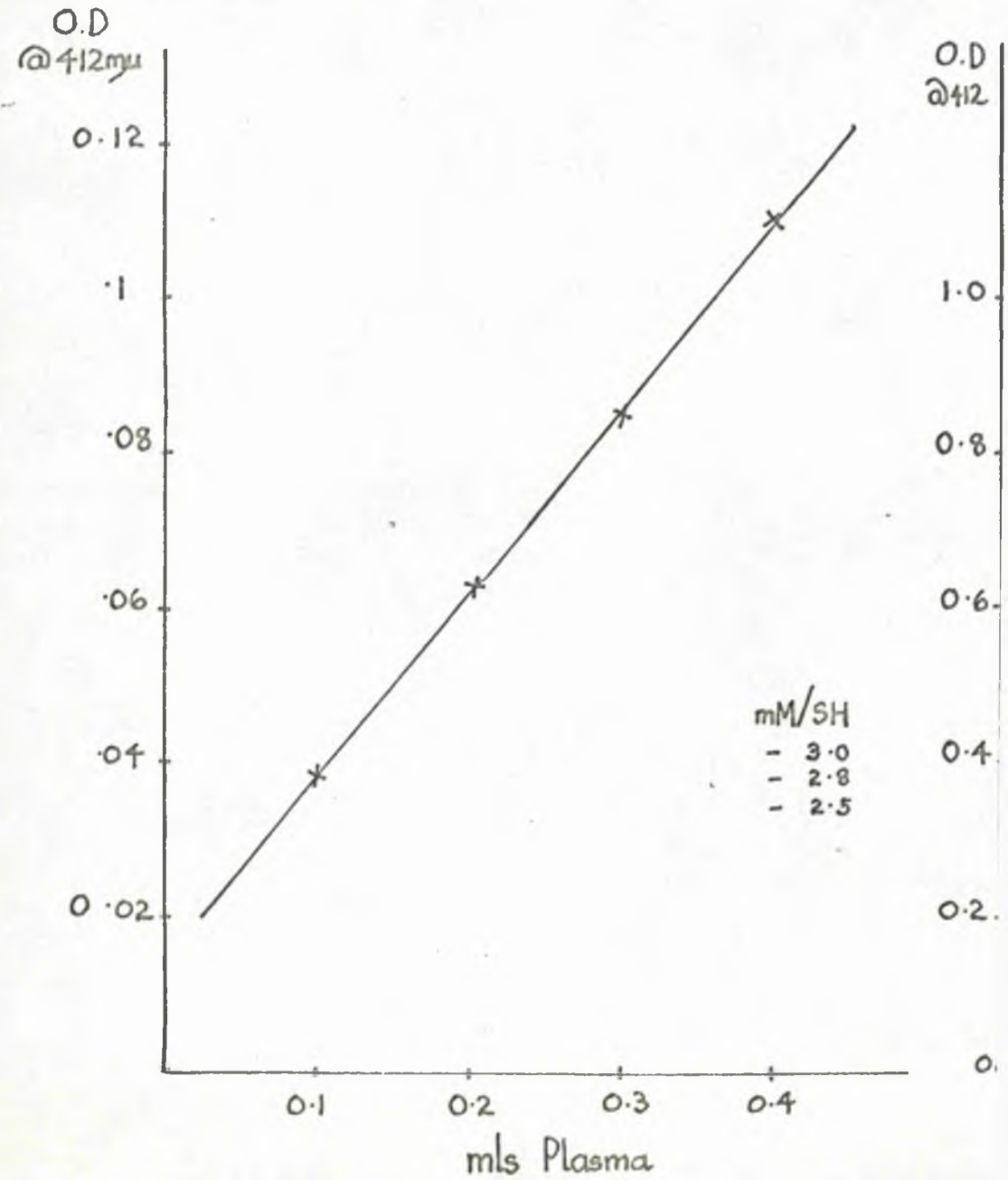
5.9 ml Water and

3 ml Acetone was added for the blank, and for the test solution 2.7 ml Acetone and

0.3 ml Reagent (PNPD).

More constant results were obtained if the plasma is added last of all after the other reactants have been carefully but thoroughly mixed. The results of increasing volumes of

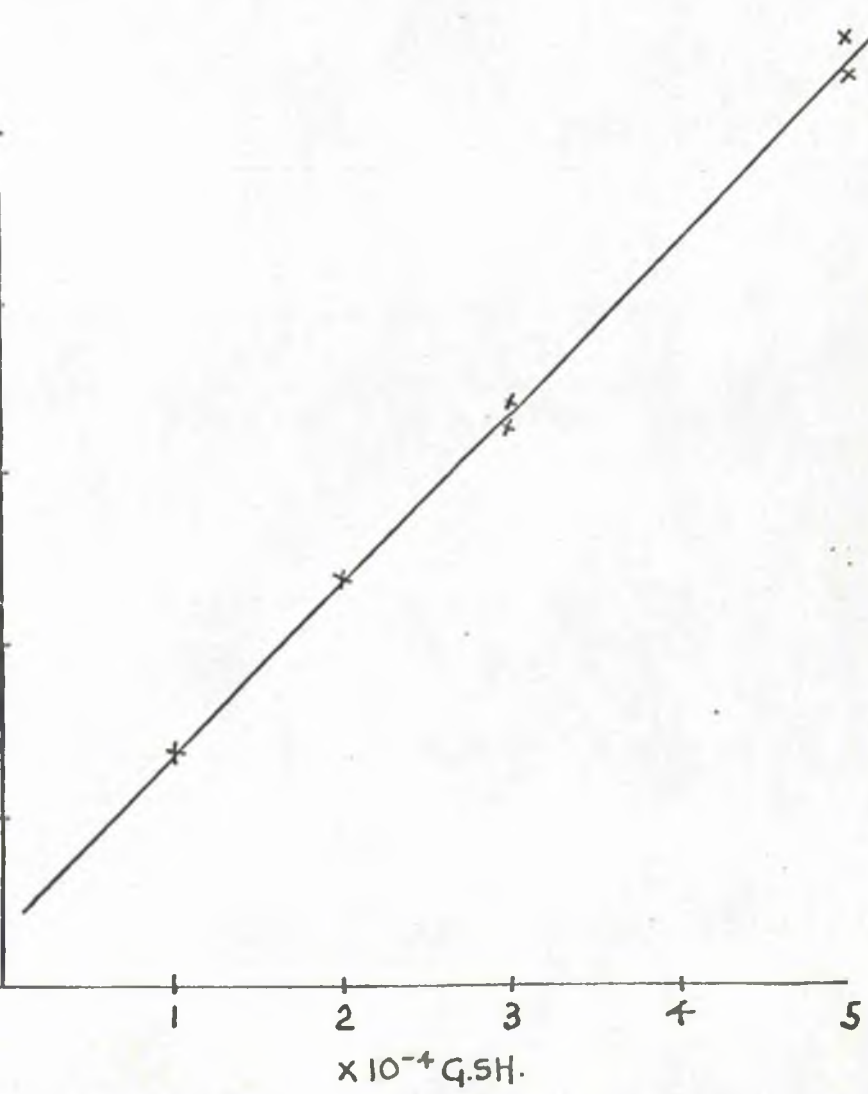
FIG. 2D·3 p. nitrophenyl anion liberation
a. Plasma



with

b. Glutathione

$1{\cdot}0 - 5{\cdot}0 \times 10^{-4}$ M



plasma are seen in Fig. 2D.3. The sulfhydryl content of normal plasma was worked out on at least 5 different 0.1 ml aliquots and ranged from 2.5 - 3.0 mM/SH/L.

Discussion.

The results complement all those found with the DTNB method.

The fact that a volatile solvent has to be used for the reagents is a disadvantage since these have to be made up at frequent intervals.

The use of acetone also gives rise to Schlieren lines in the photometer cells.

The most important disadvantage however, is the fact that not only are reactive sulphydryl groups detected, but due to the profound effect of its denaturing ability all or a large number of the more unreactive groups are also assayed.

Thus the use of PNPD is restricted to the detection of total and not just reactive sulfhydryl groups and thus by its nature is of no use in detecting small changes of denaturation occurring in blood and plasma samples from open heart surgery.

The fact that it is the insoluble relative of DTNB has been mentioned.

CONCLUSION.

1) PNPD is the water soluble derivative of DTNB already mentioned.

2) The use of PNPD is restricted to the assay of total - SH, since the denaturant - acetone - is used in the reactant

mixture. Thus the increase in - SH content of operation blood samples due to minimal denaturation cannot be assayed.

3) It may be used with equal success on both plasma and blood samples in which total - SH content is required.

SECTION 2 E.

INTRODUCTION

A Nitroprusside method for sulfhydryl detection.

In the preliminary work of selecting reagents for routine - SH assay of blood samples the following nitroprusside method of Grunert et al., 1951, was used. However due to factors which will soon be apparent the method was discontinued.

A Nitroprusside method for estimation of Glutathione.

It has been found (Grunert et al., 1951), that the detection of the sulfhydryl group in glutathione by nitroprusside and sodium carbonate leads to rapid colour loss in minutes. The addition of the cyanide radical however has been claimed to stabilise this immediate colour formed.

This claim was tested but was not found to be substantiated.

Method.

0.25 mls fresh blood was diluted and haemolysed with 0.5 mls water and a pinch of saponin. The protein was precipitated by

1.25 mls 3% Metaphosphoric acid (sat^{d})

↓

Mixed well + Centrifuged.

ppt. ← → Supernatant.

↓

2 mls taken and mixed with

6 mls saturated sodium chloride.

Both the test solution and the blank were equilibrated at 20°C for 5-10 mins, then

1 ml sodium nitroprusside (0.067 M)

1 ml (sodium carbonate (1.5 M)
(sodium cyanide (1.5 M)

was added, thoroughly mixed and the absorption immediately read using a 404 or 624 filter.

The results are seen in table 2E.1.

Not only is the colour not stable but widely differing results from standards are seen.

The ability of cyanide to reduce the -S-S- linkage to the - SH form suggests that this method would determine total rather than reduced glutathione, but as this reaction is carried out under alkaline conditions the reducing action of the cyanide is absent. Therefore only reduced glutathione is being measured. Cysteine and ergothionine in the blood however interfere with this assay.

In normal blood there is a mean concentration of 35 mgm/100 mls glutathione this is equivalent to 87.5 µgms/0.25 mls blood. Although 100 and 200 µgms GSH were added to this volume of blood there is no apparent relationship in the various results obtained, e.g. one blood sample gave a higher absorption on its own than one containing 200 µgms GSH.

The O.D. range of blood values (0.125-0.225) included those containing 100 and 200 µgms glutathione.

The method was no longer pursued as more recent, more specific and more reliable reagents are available for determination of sulfhydryl groups.

TABLE 2E.1.

	200 µg. GSH + 0.25 ml Blood.		
	0 min.	0.185	
	2 "	0.177	
	4 "	0.175	
	6 "	0.165	
	100 µg. GSH + 0.25 ml Blood.		2 different samples.
	0	0.15	and 0.225
	Blood 0.25 mls.		
1.	0 mins.	0.125	
	3 "	0.115	
	5 "	0.105	
	10 "	0.098	
2.	Blood 0.25 mls.		
	0 min.	0.225	
	3 "	0.198	
	5 "	0.181	
	10 "		
3.	Blood 0.25 mls.		
	0 mins. =	0.175	

CONCLUSION.

1) Due to the absence of - SH specificity and unreliable nature of the nitroprusside method the use of this reagent was rapidly terminated.

SECTION 3.

Turbidity. INTRODUCTION.

The turbidity of a protein solution increases with denaturation. This may be measured by determining the absorption at 620 mμ using water as a blank. It is a simple procedure and lends itself very readily to assay when the spectrophotometer is being used for more exacting techniques such as sulfhydryl determination.

All plasmas' after a fatty meal give high absorptions at 620 mμ due to the high lipid content. This apparent artefact of course is negligible in plasma samples from patients at operations for two reasons:

1) The patients are fasted prior to operation, and
2) Any blood which has to be administered during operations is usually of small volume.

It must be added however, that blood donors may yield blood of varying lipid concentrations - although this is probably mainly of low levels. This possible artefact is minimised by assaying the turbidity of a control plasma at operation and noting times of administered blood.

Recently it has been reported by Correll 1969, that electrical stimulation of certain parts of the brains of cats and dogs produced marked changes in the turbidity of the plasmas. Animals under stress - spontaneously enraged or frightened also showed a sudden marked lipaemia. During stimulation the mean

increase in plasma total lipid concentration was 63 mgms ± 15 mgms/100 mls. It is of great interest that the anticoagulant effect of heparin is decreased by cerebral stimulation (electrical) suggesting the release of an anti-heparin factor. The lipaemia clearing effect of heparin also diminishes on stimulation. It is suggested that these findings may throw light on stress related cardiac disturbances, and also the aetiology and pathogenesis of atherosclerosis.

Since stress is present at operation it may well be suggested that besides turbidity changes being a parameter of denaturation, they may also be a qualitative indicator of the degree of cephalic stimulation as a result of this stress. Whether this increase in lipaemia is due to increase in plasma lipids due to denaturation of lipoproteins or due to a decrease in the lipaemia clearing effect of heparin is difficult to assess at this stage.

Since the importance and implication of these qualitative changes cannot be blamed totally on denaturation, these changes have been relegated to a separate section in their own right.

Methods.

Half-hourly plasma samples taken from operations of 17 patients were screened undiluted, for absorption at 620 mu. The turbidity absorption of the Melrose experiment was also assayed over the 8-9 hours procedure.

Results.

The results of the operations and Melrose experiment turbidity values are seen in fig. 3.1. The composite graph shows the mean values from 17 patients taken at half hourly intervals throughout the respective operations, together with the maximum and minimum values.

The values of all the operation samples give very consistent results in 1) optical density values and

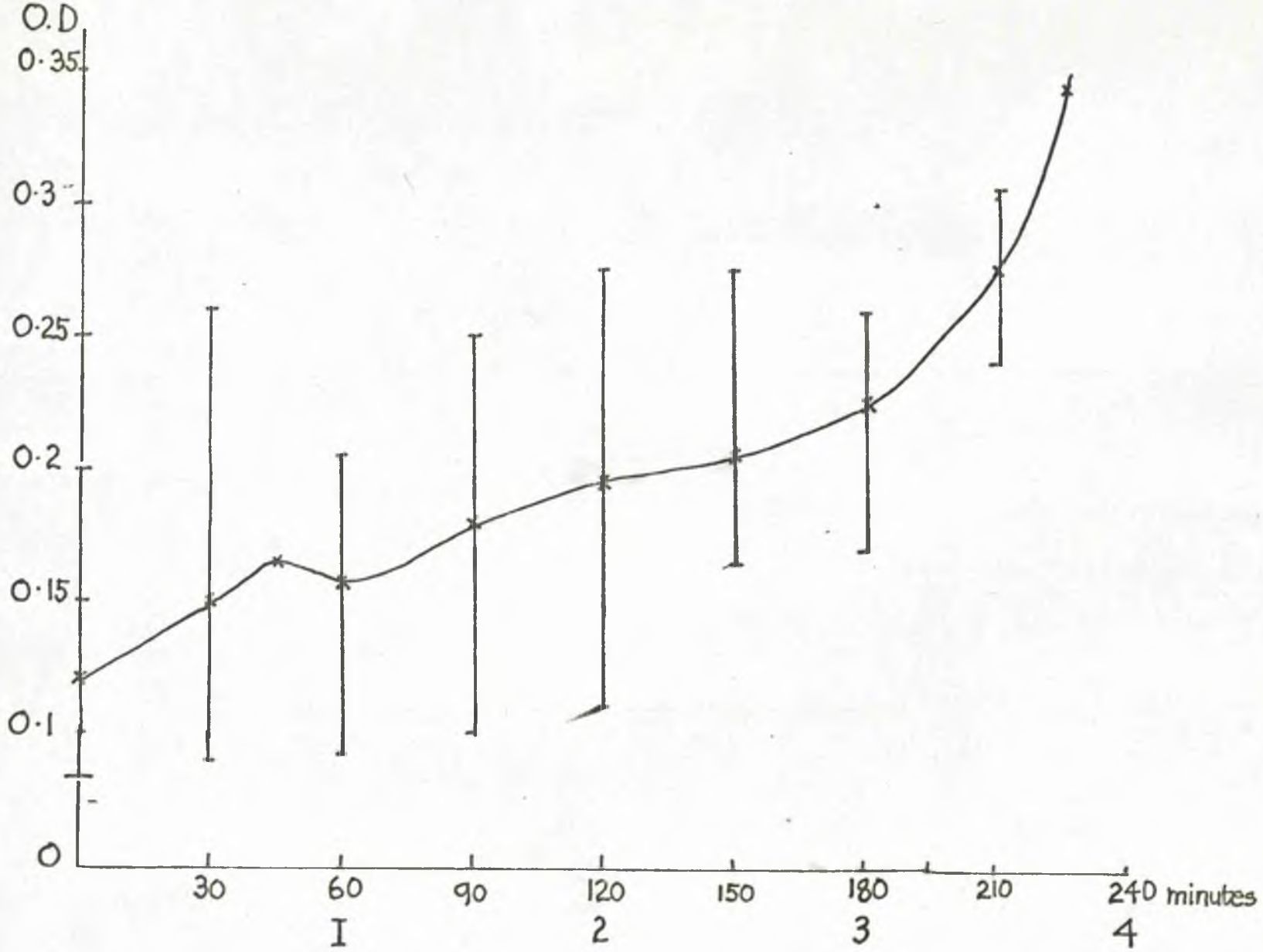
2) the increase in absorption over varying periods of time.

Whereas the operations give absorption values between 0.1 and 0.35, the Melrose experiment gave remarkably high results. The control value was 0.525 and increased considerably to 1.3 after 9 hours on the Melrose closed circuit.

Although the plasma turbidities of operation 'I J M' were assayed, the results were not included in the mean, maximum and minimum values of fig. 3.1 owing to the large volume of additional blood which was necessary in this case. These results may be seen in the corresponding table however. In this operation 21 pints of blood was added during full perfusion which lasted 4 hours approximately. Although this extra blood was added more or less consistently during the operation, the turbidity values show a definite decrease during the 2-3 hour period.

The control general surgical operations showed results very similar to and in the maximum-minimum range of the cardiac

FIG. 3·1 Change in Plasma turbidity during full perfusion. Mean values



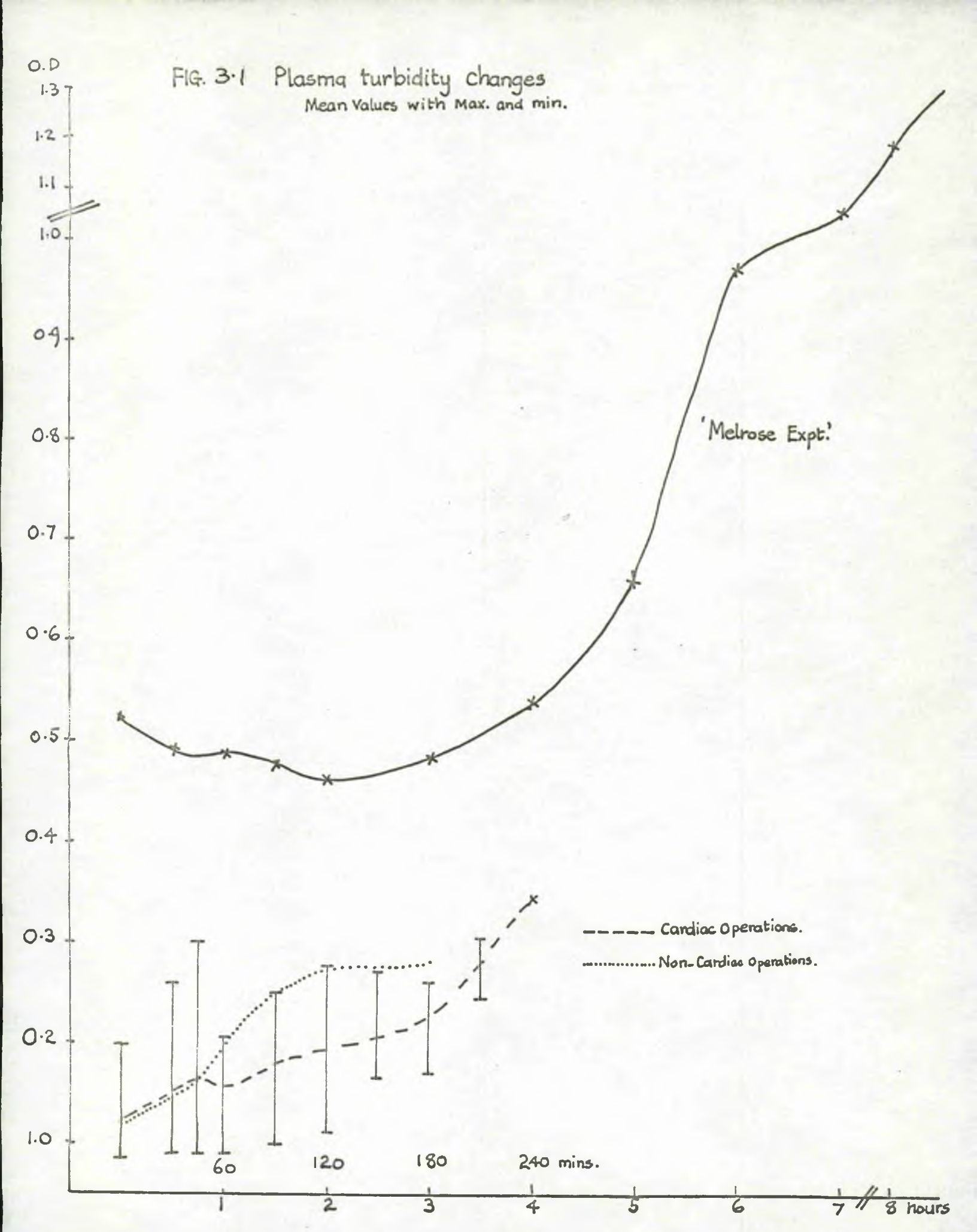


FIG. 3·1 Plasma turbidity changes
Mean Values with Max. and min.

operations.

Since the exact importance and implication of these qualitative changes are open to some speculation, it has been thought sufficient to represent the results on one graph rather than eighteen.

DISCUSSION.

All the plasma turbidity cardiac results show an increase which is roughly proportional to the duration of full perfusion i.e. total oxygenation by means of the Ryggs bag. The fact that the control operations show virtually the same change indicates that this turbidity increase is a non specific effect probably due to the stress and trauma of operation. If blood and diluent were not added at all during operation (assuming minimal blood loss) these results would show even greater changes.

The fact that there is an increase in turbidity when minimal volumes of blood or diluent is being added during operation suggests that either or both of the following are taking place:

1) increase in plasma lipids either by direct addition to blood or by breakdown of plasma lipoproteins.
2) decrease in lipaemia clearing factor (heparin), - in excess of the rate of dilution.

Since heparin is constantly given (one hourly) throughout perfusion it would a pear that an overall increase in plasma lipids is the more important factor, query due to

cephalic stimulation by stress.

All the operations show a similar trend except "IJM" in which a turbidity decrease is seen during the 2-3 hour period - this was obviously due to dilution in excess of the rate of lipaemia increase.

The high initial value of the Melrose experiment is possibly due to slightly less dilution being used than is usually the case in perfusion volumes, although care was taken in the Melrose experiment to keep operation conditions as standard as possible. The blood used in this experiment was only 2-3 days old, - the same age blood as used in perfusions. It must also be borne in mind that in perfusions the perfusate volume is mixed with the even greater blood volume of the patient. Another possibility in this "turbidity difference" is that the donor blood may well have been in a post prandial "fish and chips" state.

Control plasma's from students in the pre prandial state gave turbidity values similar to control operation results.

The fact that the turbidity rises in the Melrose experiment after 2-3 hours may be due to loss of heparin during the run owing to it becoming inactivated over the 9 hours at 37°C. Thus the increase in turbidity of plasma in operations seems to be mainly due to actual lipid increase - ? protective role, and in the Melrose experiment to heparin destruction. Owing to the considerable expense of using fresh banked blood for research purposes only, it has not been

possible in this series to repeat the "Melrose experiment" using a Rygge Bag.

The control non cardiac operations showed a slightly faster increase in turbidity values over the first two hours but was within the range of perfusion results. The small differences being explained on minor blood volume variations.

CONCLUSION.

1) Plasma turbidity increases proportionately with duration of perfusion.
2) General surgery gives rise to a similar increase in plasma turbidity thus indicating that it is due to stress and trauma of surgery in general and not to oxygenation of blood in particular.
3) The major cause of increase in turbidity is probably due to cephalic stimulation (by stress factors) resulting in one or several of the following:-
    (a) Release of lipids directly into the circulation
    (b) Breakdown of plasma lipoproteins (? by denaturation)
    (c) Decrease in the lipaemia clearing factor (heparin).

Plasma Viscosity. Introduction.

The viscosity of a protein increases with denaturation. This was measured in a capillary Viscometer using undiluted plasma (13-14 mls) at 20°C, and compared with water. The viscosity of a solution (η) may be measured in centipoises and is given by:

η/η_o = t/to = d/d_o = relative viscosity

where η_o = viscosity of solvent (water)

t and t_o = time of plasma and water respectively (secs)

and d and d_o the density do. do. do. do. do.

The specific viscosity = Relative Viscosity -1

$$= \frac{\eta - \eta_o}{o}$$

It is interesting that Wright et al., 1962, express Viscosity in Staudinger units

$$= \frac{t/t_o - 1}{C}$$

where C = protein concentration in gms/ml.

Of all the work done on the investigation of denaturation of plasma proteins by various agents perhaps the most intensively studied is albumin.

Kauzmann et al., 1953, has shown that bovine plasma albumin undergoes an immediate increase in intrinsic viscosity on exposure to urea solutions in concentrations above 2 $\bar{M}$. This change is instantaneous and is also reversible so long as the temperature is kept below 40°C.

The change in this viscosity of albumin is similar to these changes which take place at lower pH in the absence of urea.

Saenko 1952 investigated denaturation of plasma albumin by heat and found that the intrinsic viscosity increase could be prevented if aggregation of the molecules was minimised by utilising conditions of either 1) High net charge

2) low ionic strength and

3) low protein concentration.

In a stable environment any denaturation of plasma proteins would be expected to show an increase in viscosity. However it became increasingly obvious throughout these perfusion studies that other factors leading to increased viscosity had to be considered as well; these were

1) Variation in blood volume and constituents during full perfusion, i.e. the degree of haemoconcentration or haemodilution.
2) The effect of the oxygenation (trauma) on the red blood corpuscles. It has been shown that damaged corpuscles leads to decreased capillary flow, mainly through their becoming 'sedimented' around the periphery of the capillary walls, and thus called "sludging". This latter phenomena has been observed in alcoholic hang-overs and minor respiratory disease as well, Lee *at al*., 1961.

Although the ratio of the blood constituents was kept very stable during perfusion, inevitable losses and small blood volume (and occasionally dextrose/saline) additions had to be made.

Thus it would appear that viscosity increases are due to the following factors.

1) The overall temporary increase in haemoconcentration due to fluid loss in excess of replacement

i.e. (a) operation site

(b) urine

(c) lungs and skin

2) The increase in free haemoglobin in plasma (haemoglobinaemia) - other products of red corpuscle damage may also be present,

3) the denaturing blood proteins,

4) due to stress and duration of operation factors (proteins, toxins) may well be liberated in the blood itself and also liberated into the blood from damaged cells and tissues.

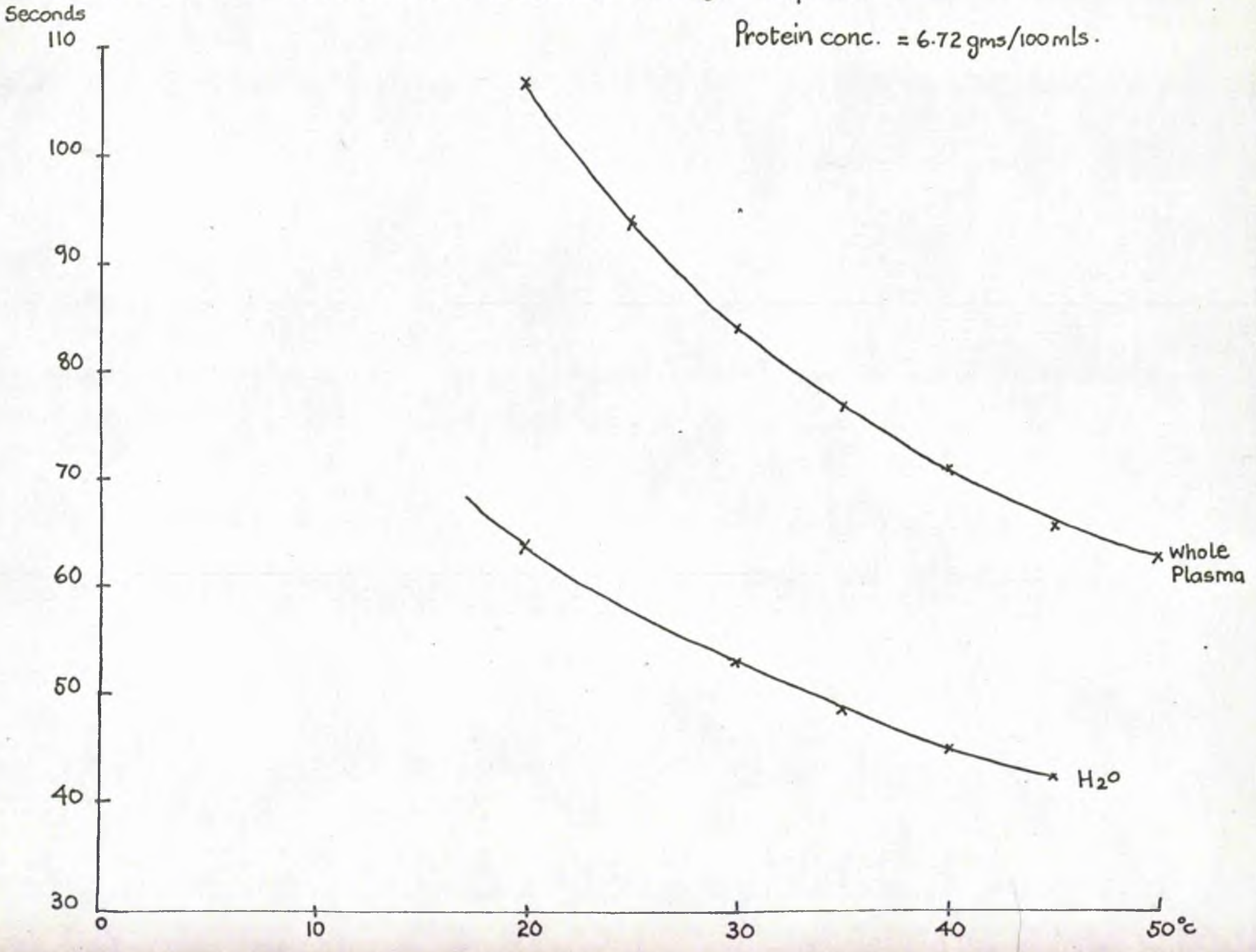
Wright et al., 1962 have shown that viscosity increases during perfusion but no comment is made on the significance and the participating factors of this change.

As viscosity changes during perfusion are multifactorial in origin and denaturation probably plays a minor role, viscosity has been given a section of its own rather than included as a subsection of denaturation.

Method.

The standard capillary viscometer was used throughout

FIG. 4·1 The relation of Plasma and Water Viscosity to temperature.



all the perfusion studies although initially the Brookfields Synchroelectric Viscometer was used with rather equivocal results.

The capillary viscometer held a volume of approximately 13 mls and the viscosity was determined of plasma at 20°C. Successive measurements were done until readings were obtained within 0.1 seconds.

Results.

At 20°C whole normal fresh plasma and water gave mean values of 106.5 and 63.7 seconds respectively using the capillary viscometer. The concentration of protein in this plasma was 6.72 gms/100 mls, fig. 4.1.

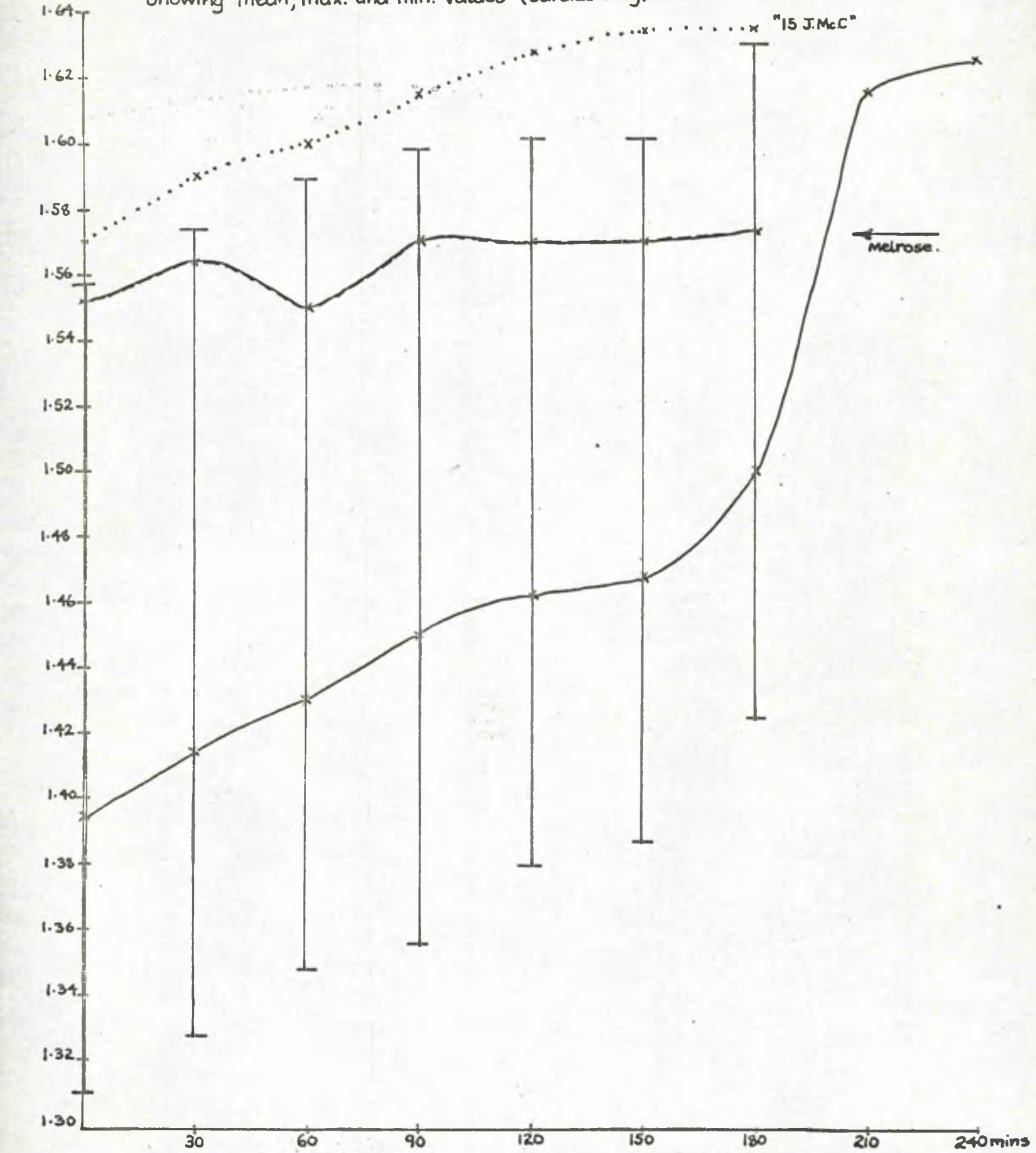
The relative viscosity is thus 1.672 c.p.s. and the specific viscosity 0.672 c.p.s. The specific viscosity of water at 20°C is 0.5608.

The viscosity results (in t seconds) of serial plasmas from operations varied between 84 and 106 seconds depending on the protein concentration, which in turn was obviously due to the degree of dilution in the total perfusate volume.

The results therefore of the plasma viscosities may only be compared directly with other results from the same operation. Although qualitative differences may be obtained from results of other operations, i.e. Type and shape of the Viscosity curve.

The plasma protein concentrations varied between 4.4 and 6.5 gms/100 mls. The mean value was approximately 4.8

FIG. 4·2 Change in relative viscosity of plasma during operations.
Showing mean, max. and min. values (cardiac only)



gms/100 mls. A relationship was observed between the plasma flow in "t" seconds and the protein concentration. Approximately the plasma flow decreased (i.e. time t increased) 5 seconds for every 0.5 gms protein increase.

The mean value (t seconds) of the viscosity increase of the 5 short cases - 30-45 minutes - was 1.04 seconds.

The mean value of the 2 medium length operations - 2 hours - was 3.7 seconds, and the mean value of the nine 3-3½ hour operations was 5.66 seconds.

Thus it is clear that the viscosity of plasma increases with duration of operation.

These viscosity changes are at the best only qualitative owing to the various influencing factors. The results have been expressed directly in centipoise units.

DISCUSSION.

The results are seen in table 4.1 and fig. 4.2. 16 patients are represented. In the last case, '17', insufficient plasma was available without considerable dilution being required and thus the viscosity was not determined, of the 16 patients remaining, one, '15 J Mc', was the oesophageal carcinoma/hiatal hernia patient. The remaining 15 patients are subdivided into 3 sections of short, medium and long perfusions as seen above.

It is also clear on analysis of the above figures that although the viscosity increases as the plasma protein concentration increases the latter is by no means responsible

for the total increase in viscosity. This fact emerges in operations in which no extra blood was added and in which the plasma protein concentration remains fairly constant throughout the full perfusion, e.g. "3 B.K." and "5 I.D.".

There is one other factor which may well affect these viscosity results and this is the increasing concentration of free haemoglobin. The effect of this could be studied by adding controlled amounts of haemoglobin to fresh unhaemolysed plasma, correcting for dilution and noting the viscosity change.

From the above table the graphs of the increase in viscosity of individual cases does not follow any regular pattern. Some graphs are straight, others sigmoidal or curved. This is due to several factors:

1) Whether or not extra blood is utilised in the operation
2) Whether or not dextrose/saline " " " "
3) The degree of haemolysis.
4) To a lesser extent - the duration of the operations.
5) The degree of haemoconcentration (or dilution) during perfusion.

In most cases the plasma proteins increase in concentration during perfusion but in two particular cases "16.K.R." and "11. A.C" there were distinct falls.

In "16 K.R." the protein fell from 7.0 → 5.7 gms/100 mls plasma over a 45 minute perfusion. This patient was a 5 year old boy and thus it is easy to account for sudden

protein change while trying to maintain correct electrolyte balance, blood volume etc.

The second case "11. A.C" was a 43 year old lady. Here a plasma protein fall from 4.7 → 4.1 gms/100 mls was observed over a 3 hr. perfusion. This was probably due to more than usual loss of albumin at the operation site, since blood diluents were only very sparingly used during this full perfusion. The plasma albumin dropped from 2.9 to 2.4 gms %.

The results of "the Melrose experiment" are interesting for over the 6 hour period when blood and diluent was continually circulated through the Melrose machine only an overall minimal increase in viscosity was observed. The viscosity of these plasma samples (6) was also obtained using a Brookfield synchro-electric viscometer at constant temperature 20°C. This apparatus works on the principle of a rotating disc which is placed on the meniscus or in the solution to be tested, and the torque necessary to overcome the viscous resistance is measured and converted into centipoises. Although this gave rather equivocal results, they tend to support the findings using the capillary viscometer. A criticism of the synchro-electric viscometer is that in spite of a theoretically temperature controlled environment, the spinning disc on contact with the plasma momentarily produces heat which obviously affects the viscosity.

The control oesophageal resection and hiatal hernial

repair operation "15 McC" also showed an increase in viscosity. This was 4.15 seconds for the 3 hour operation which is below the mean value for open cardiac operations of this duration (5.66 seconds).

CONCLUSION.

1) The viscosity of plasma increases with duration of perfusion. This is probably due to not only concommitant haemoconcentration (relative plasma protein increase) but also to
   (a) increasing haemolysis.
   (b) the addition of extra blood to the perfusate volume.
   (c) ? plasma protein denaturation.
   (d) The addition to the circulation of unknown ? toxic substances.
2) The viscosity increases in the control operations (general surgery) were slightly less than the perfusion results.

SECTION 5.

Haemolysis

Introduction

As mentioned in the introduction and denaturation chapters, the passage of blood through the extra corporeal circulation leads to progressively increased haemolysis. The rate of this must be measured and a reliable method will be described for serial blood samples.

The relation this has - if any - to the increase in blood amino acids will be discussed later when the amino acid data from these operations is also discussed.

Methods.

The method used to assess the degree of plasma haemolysis was a modification of the Cyanomethaemoglobin method normally used for Haemoglobin estimation (blood).

Haemoglobin in the presence of ferricyanide is converted (oxidised) to Methaemoglobin which is reacted with potassium cyanide to form cyanomethaemoglobin. This is a stable substance with a maximum absorption of 540 mμ.

$$Hb \xrightarrow{K_3Fe(CN)_6} Met\ Hb \xrightarrow{KCN} CN.MET.Hb.$$

The Haemoglobin reagent used for this conversion consisted of

1 gm sodium bicarbonate 0.2 gm potassium ferricyanide 0.05 " potassium cyanide	1 L.

This was made up to 1 litre with water and stored in a brown bottle at 1-2°C. It is stable for many weeks e.g. 6-8 weeks.

Standardisation of Haemoglobin. Wongs Method.

Before the Cyanomethaemoglobin method is used, an Iron standard must be prepared.

Haemoglobin contains 0.34% Fe.

Blood sample - unknown iron content

I. 0.5 mls oxalated blood is placed in a clean iron free 50 ml volumetric flask.

2 mls Iron free concentrated sulphuric acid was added and mixed for 1-2 minutes.

2 mls saturated potassium persulphate was added and the mixture diluted to roughly 25 mls.

2 mls of 10% sodium tungstate was then added, mixed well, cooled, and diluted to 50 mls exactly with water. This was filtered through dry paper into a dry flask.

II. The Haemoglobin Standard. Iron solution was prepared (see Appendix I) and contained 0.1 mgm ferric iron/ml.

III. The blank consisted of the same as the standard but contained no iron (see Appendix I).

10 mls of each of the above solutions (unknown blood sample, Haemoglobin Iron Standard and blank), were put into 3 test tubes respectively. To each was added 0.5 ml sat potassium persulphate solution and 2 ml 3N potassium thiocyanate.

The solutions were well mixed and the absorption taken within 30 minutes at 480 mμ, against the blank.

The determination of the Haemoglobin in the unknown

sample is given by

$$\frac{\text{Absorption of unknown}}{\text{Absorption of standard}} \times 0.25 \times \frac{100}{0.5} \times \frac{1}{3.4} = \text{gms Hb/100 mls blood.}$$

The purpose of the concentrated sulphuric acid and potassium persulphate is to detach the iron from the haemoglobin molecule. 98-99% of blood iron is in combination with Haemoglobin.

Absorption of standard was 0.75 which is equivalent to 50 mgm % blood iron = 14.7 g % Hb.

Haemoglobin content of blood: Cyanomethaemoglobin method Figs. 5.2 and 5.3

0.02 mls blood was gently added to 5 mls Haemoglobin reagent and mixed well.

The absorption at 540 mu was read after 10 minutes. The Haemoglobin solution being used as the blank.

The absorption obtained was compared with the Haemoglobin (Fe) standard result from above, thus

$$\frac{\text{Density of unknown}}{\text{Density of standard}} \times \text{gm \% Hb standard} = \text{gm Hb/100 mls.}$$

Normal Range for males 13.5 - 18.0 g/100 mls)
) Whole Blood.
Normal Range for females 11.5-16.5 g/100 mls.)

Haemolysed plasma Assay: Calibration.

The calibration of haemolysed plasma was simply performed by taking a volume of blood 98% Hb and adding exactly the same volume of water. The solution was mixed well and carefully (to avoid damage to the plasma) and then centrifuged.

0.1 ml of supernatant was added to 5 mls Haemoglobin reagent and the absorption taken at 540 mμ after 10 minutes. This result was taken as the 100% haemolysis figure. The blank consisted of haemoglobin reagent only.

The mean value of 100% haemolysis (O.D. 0.745) was derived from 5 separate estimations using fresh Haemoglobin reagent and correcting the haemoglobin content of the blood sample taken, to 100% Haldane. The haemoglobin content of the samples were estimated by the previous method.

A similar volume of blood was taken and diluted carefully with exactly the same volume of 0.9% sodium chloride. The solution was very carefully and completely mixed and then centrifuged.

0.1 ml of the supernatant was added to 5 mls Haemoglobin reagent and the absorption obtained taken as the zero level of haemolysis of plasma. The results are seen in Fig. 5.4.

The plasma samples from operations were screened for the degree of haemolysis by taking a small volume of the blood, diluting and mixing carefully with exactly the same volume of isotonic sodium chloride. The solution was then centrifuged immediately, and the supernatant removed for the haemolysis estimation.

0.1 mls of the supernatant was added to 5 mls of the Haemoglobin reagent and after careful but thorough mixing the absorption was read after 10 minutes at 540 mμ with the reagent

as blank. Virtually the same results are obtained by diluting the plasma, after initial centrifuging of blood, with the same volume of isotonic saline.

RESULTS and DISCUSSION.

By the Haldane standard there are 14.7 gms Hb/100 mls blood. Total haemolysis of blood whose Hb value is accurately known yields a mean optical density of 0.745 at 540 mμ using the Cyanomethaemoglobin method.

Therefore an accurate calibration of the degree of haemolysis can be obtained, see Fig. 5.4.

It can be seen that for every 0.01 increase in absorption there is an increase of approximately 200 (198.6) mgms of free haemoglobin in plasma. The initial mean values of both the perfusions and control operations are very close and are equivalent to a haemolysis value of 100 mgms % Hb although this varies considerably at the beginning of full perfusions from approximately 40-280 mgms % Hb.

The degree of haemolysis in the first (control) specimens of blood although minimal, are above levels normally found in fresh plasma (5-15 mgms %). This is because of two factors:

1) The blood used to prime the extra corporeal circuit was 1-3 days old and thus minimal haemolysis will have started while in storage, and
2) The control blood samples at operations were never taken before 5 minutes after full perfusion had started to

FIG. 5·1 Conversion of Haemoglobin % / Haemoglobin gms/100mls.

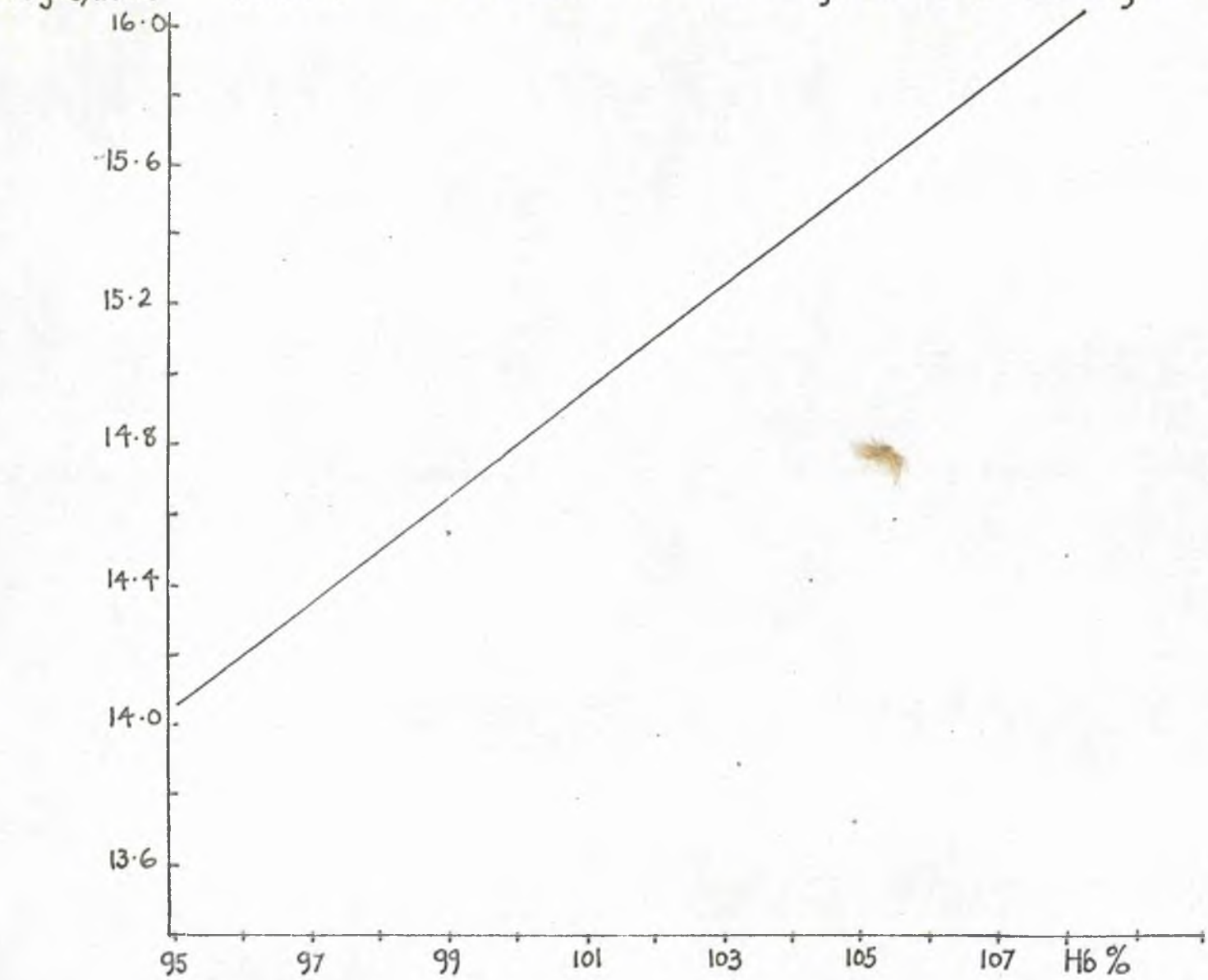


FIG. 5·2 Conversion graph of Cyanomethaemoglobin to gms % Hb.

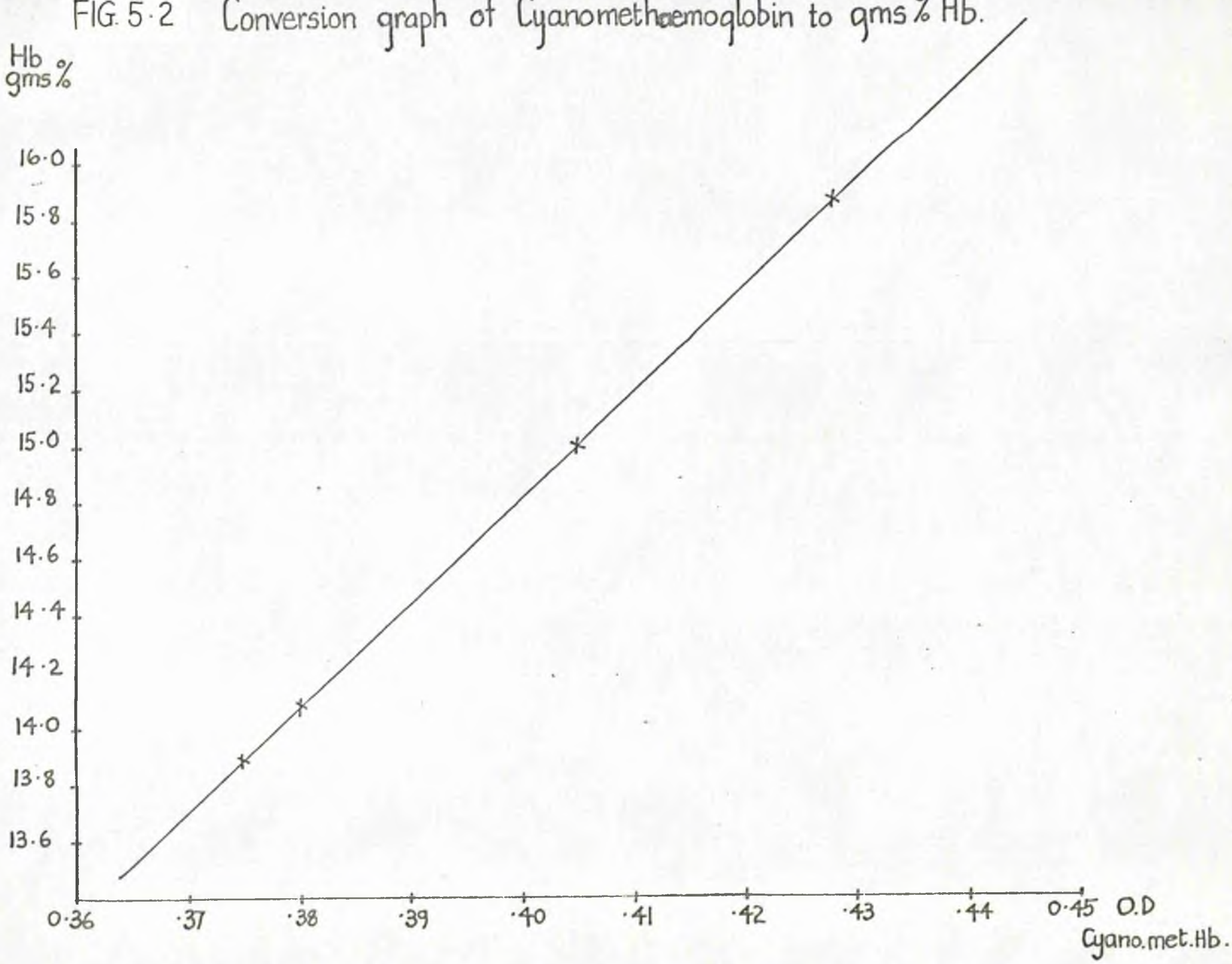


FIG. 5.3 λ Max. of Cyanomethaemoglobin : Normal blood results.

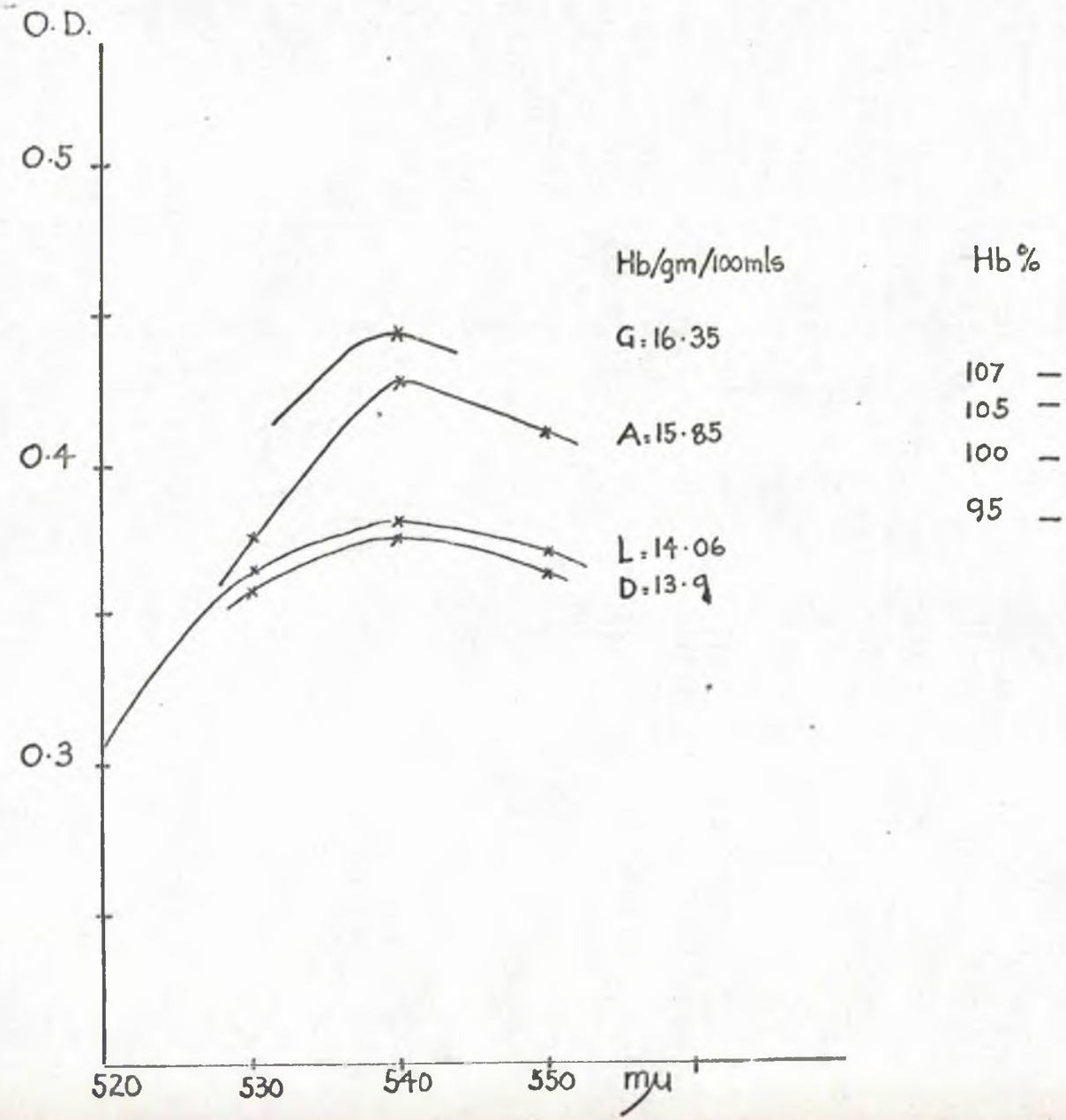


FIG. 5·4 Cyanomethaemoglobin calibration of Haemolysis @ 540 mμ

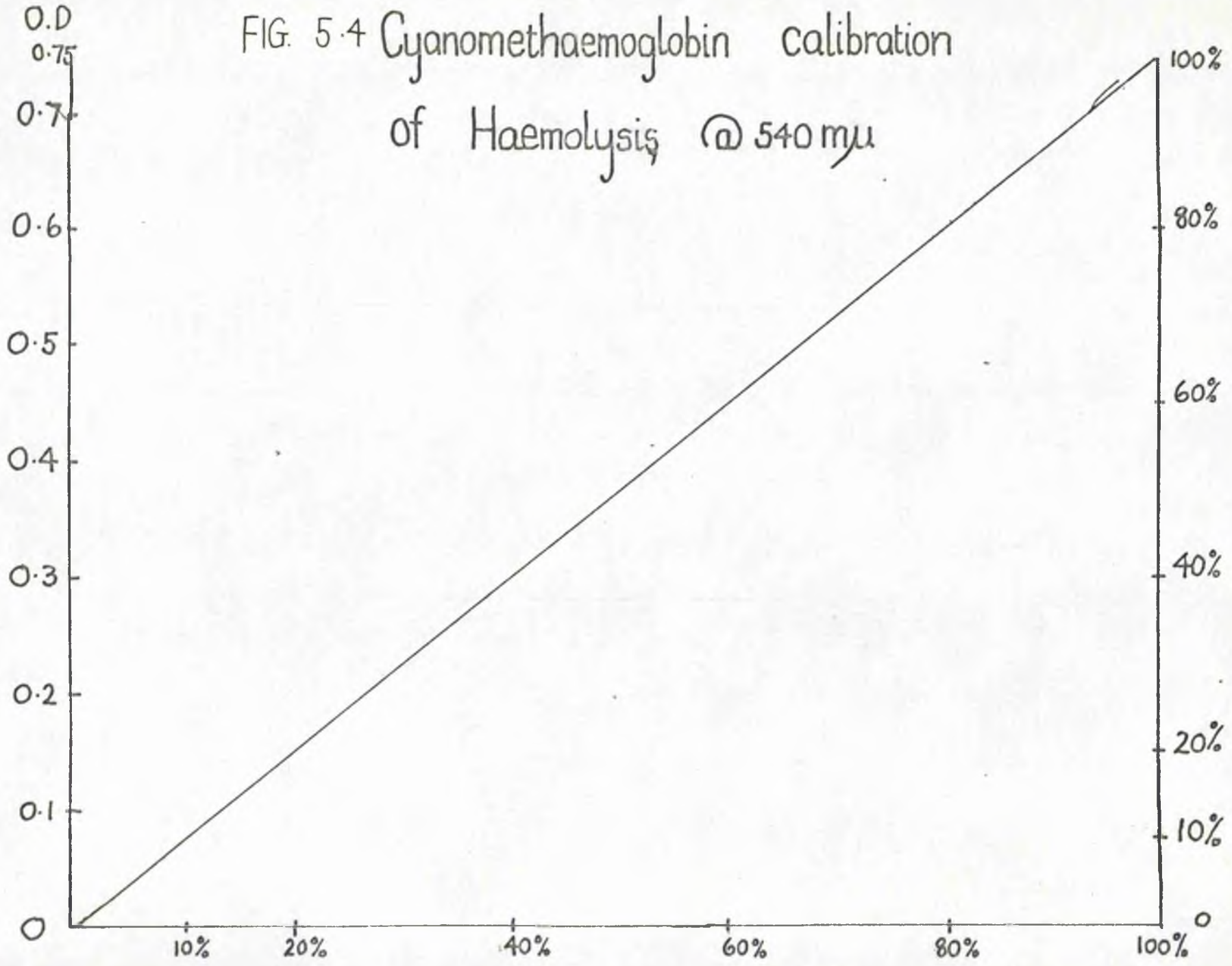
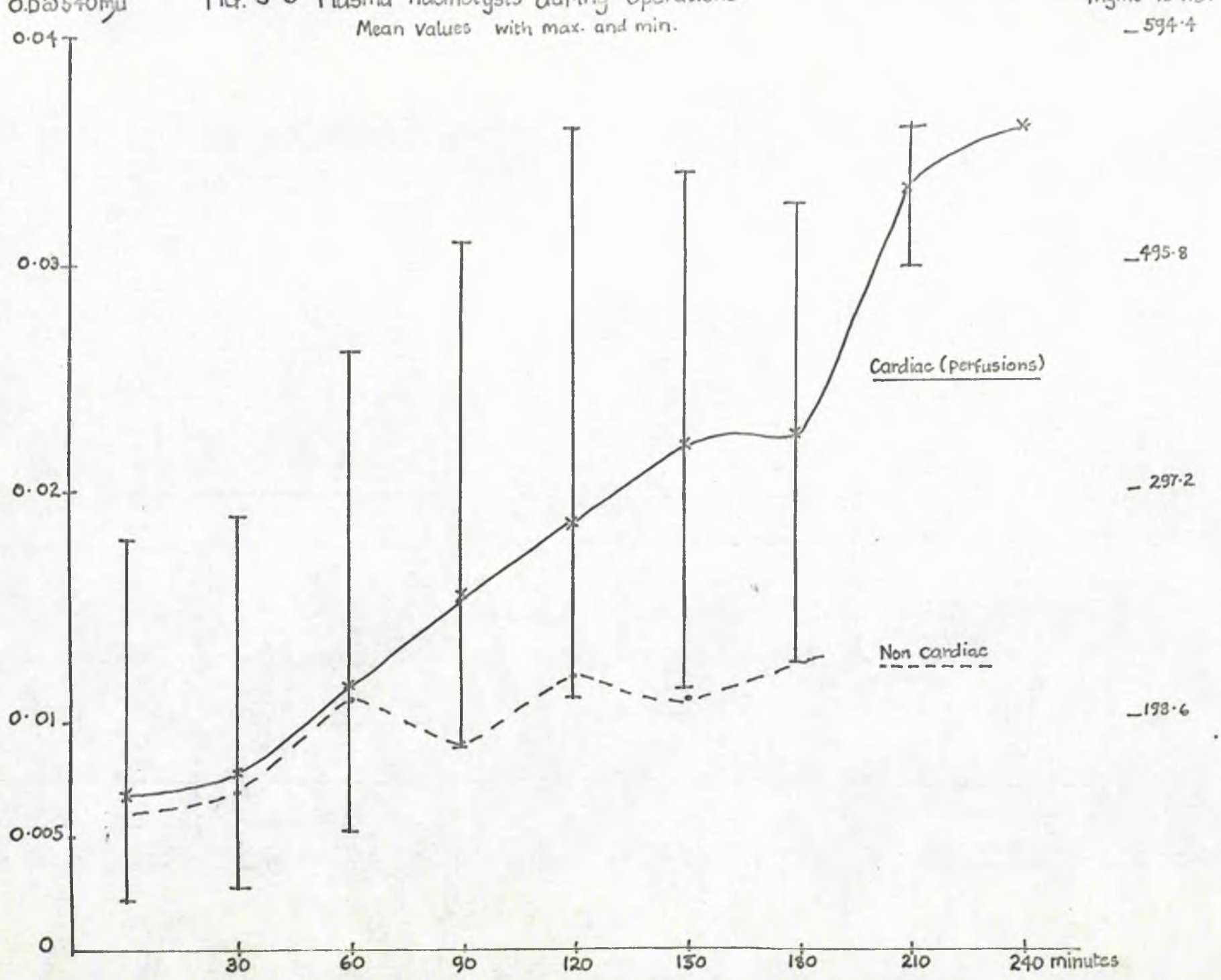


Fig. 5.5. 7 Serial samples of plasma from "the Melrose experiment" (Control) showing increasing haemolysis with time (1 hourly intervals).


O.D@540mμ
FIG. 5·6 Plasma haemolysis during Operations.
Mean values with max. and min.
mgms % Hb.
0·04
0·03
0·02
0·01
0·005
0
594·4
495·8
297·2
198·6
Cardiac (perfusions)
Non cardiac
30
60
90
120
150
180
210
240 minutes

enable complete homogeneity of blood to be attained. Thus minimal haemolysis would occur during this and the pre-partial perfusion stage.

During perfusions see Fig. 5.6 there is remarkable linearity between increase in haemolysis and duration of perfusion up to 2½-3 hours. At this point there is a 'levelling off' - which may well be an artefact due to relatively few operations of this duration to compare and thus obtain a more exact mean value. After this period there is a greater increase in the rate of haemolysis.

The control operations give haemolysis figures similar to the minimal figures seen of perfusions. Although several pints of blood had to be given in the former '15 J. Mc' only, over the 3 hour operation, it is not enough to account for the discrepancy in haemolysis rate at 3 hours between the two types of operations.

Supporting this argument is the fact that additional blood had to be slowly administered in perfusion cases as well. Thus this discrepancy is more likely to be a true representation of the haemolytic changes than apparent.

It seems clear that the rate of haemolysis occurring in perfusions is due to 1) the oxygenation method,

2) the destruction of the erythrocytes by the action of the rollers in the pump mechanism, and

3) probably least important,

? mild haemolysis due to the passage of blood through 20-30

feet of siliconed tubing and stainless steel connectors.

The great increase in haemolysis which occurred in the Melrose experiment may be seen in Fig. 5.5.

CONCLUSION.

1) Cyanomethaemoglobin is the most stable haemoglobin pigment and is ideally suited for not only haemoglobin estimation in blood itself but also to assess the degree of haemolysis in plasma, (haemoglobinaemia). A method for the latter is described.
2) There is marked linearity between the degree of haemolysis and the duration of perfusion over the first 2½-3 hour period.
3) After 3 hours there is a more rapid increase in haemolysis.
4) The general surgical operations showed close affinity with perfusion results over the first hour. After this however the increase in haemolysis is distinctly less in the former compared with the latter.
5) The main causes of haemolysis are due to
   (a) the method of oxygenation.
   (b) the effect of the occlusive rollers on the erythrocytes.

SECTION 6 A

Electrophoresis of plasma proteins

Introduction

Four different media - paper, cellulose acetate, polyacrylamide gel and starch - each under varying conditions of buffer, ionic strength, voltage and current etc. were used with varying results.

On paper - inert supporting media - proteins are separated solely by virtue of their size and electrical charge.

Cellulose acetate membrane (CAM) like paper is an example of zone electrophoresis. However, by the formers very nature it has distinct advantages over paper.

Owing to few hydroxyl groups in the membrane, it is virtually non absorptive so that separations are quick, well defined and tailing minimised. CAM is therefore a more ideal medium to use for insulin, fibrinogen histones and lysozymes than paper. Background absorption of dye is eliminated thus giving better contrast to the protein stained areas. It can also be used for immuno electrophoresis and isotopic labelling unlike paper. In addition small quantities of protein need only be used.

Polyacrylamide gel. This excellent method described in detail in this section separates proteins not only by electrical charge but also by exerting a sieve like action depending on the molecular size of the molecules. Since the molecular weight of plasma proteins only differ by a relatively small

amount (in comparison with some proteins) it is quite clear that in order to separate plasma proteins efficiently two or more different techniques must be used in their fractionation. Polyacrylamide gel does this excellently.

Starch like polyacrylamide relies on at least two factors to separate proteins. 1) A filtration effect (larger molecules move slower than smaller molecules), and 2) Separation by electrical charge. Other factors no less important are 3) Narrow starting zone and 4) Minimal diffusion of starting sample and throughout run.

Apparatus

For paper, starch and some cellulose acetate strip (20 cms) work the standard LKB Electrophoresis tank and power pack was used.

General method

Before details of the different media used are described several important general points will be mentioned.

1) In order to obtain satisfactory separation of such similar proteins found in plasma it is very important that equilibration between the tank buffer electrode wicks and the media used is carried out prior to every run. 30 minutes is a good minimum time but no harm is done by leaving equilibration for several hours.
2) The temperature of electrophoresis is important. The greater the current and voltage employed the greater the heat produced. Hence if these factors must be high the

separation must be carried out in a cold room or be cooled by other means like the method employed with High Voltage electrophoresis.

Electrophoresis may be carried out with satisfactory separation at room temperature providing the voltage is low e.g. 100 V . This often necessitates however a run lasting 17 hours or so.

The appearance of heavy condensation on the underneath of the electrophoresis cover is an indication that evaporation is taking place inside due to relatively excessive heat production. This may be corrected three ways

1) Reduction of voltage/current.

2) Removal to cold room

3) Removal of cover and wiping away excess condensate - this technique is not to be recommended since equilibrium is obviously destroyed in the separation chamber.

3) Owing to pH changes in the electrode compartments it is essential to reverse the polarity after each run.

4) The buffer levels in both compartments were checked for level, to avoid siphon effect across the wicks.

5) In preliminary runs bromophenol blue was used as a marker to give position of albumin peak during separation.

Paper Electrophoresis: Method

Barbitone buffer : 10.3 gm sodium barbitone

1.83 gm barbituric acid (barbitone)

was dissolved in 950 mls water with shaking and made up to one

litre.

pH = 8.6 Ionic strength 0.1 μ.

Paper used was 3 MM or Whatmans No. 1.

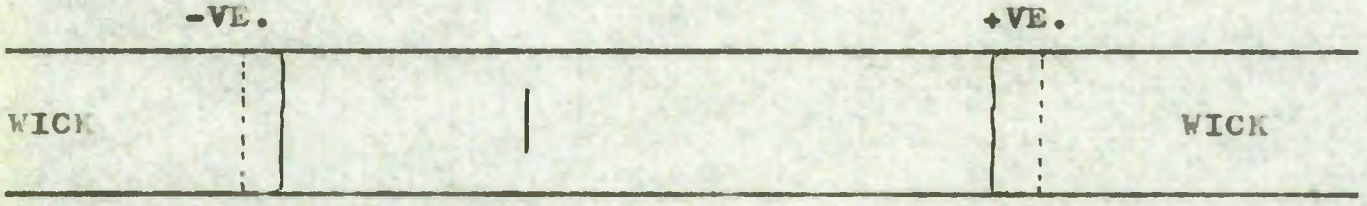
Paper strips of approximately 3 x 10 cms were normally used.

For overnight runs (16-18 hrs.) 100 Volts and a current of 0.1 mA/cm were used and the separation carried out in an environment of 1-2°C. This reduced denaturation of protein to a minimum since the heat produced at 100 volts is small even at room temperature.

The strips were dipped in buffer and lightly blotted with clean filter or chromatography paper and allowed to equilibrate in chamber as mentioned above.

4-6 μl plasma were added carefully to the origin (already marked) by the L.K.B. applicator.

In the above buffer, plasma proteins exist as and consequently migrate towards the anode. In order to get maximum separation with the minimum amount of dilution (of bands) and failing, the origin was placed approximately 3/8 of the way along the paper strip from the cathode end, thus



ORIGIN.

After separation the strips were immediately dried in an oven 100-105°C for 5-10 mins., this denatures the protein

and stops diffusion of the protein bands.

Staining of bands was done with 1) Napthalene Black 12B (Amidoschwartz) 1 gm/100 mls of 10% acetic acid in Methanol for 10 mins.

The strips were washed four to six times in fresh 10% Acetic Acid/Methanol and finally in methanol and air dried.

or 2) Bromophenol Blue

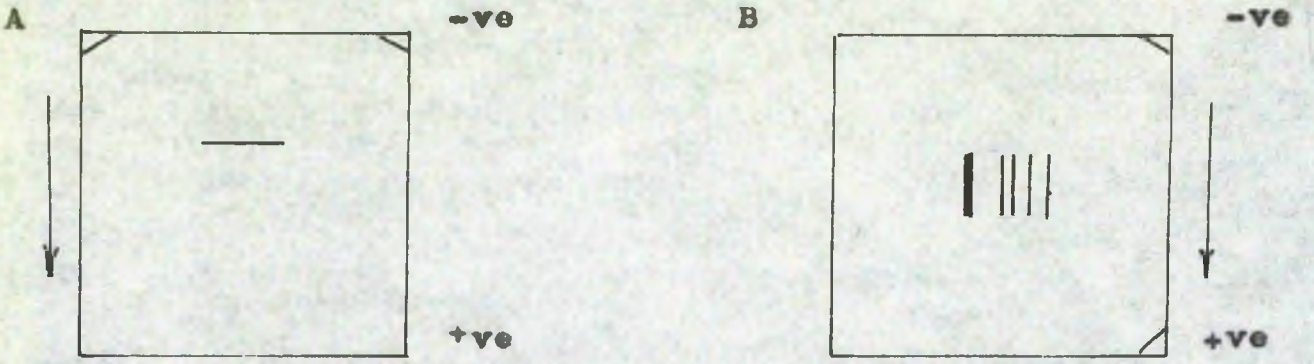
1% in 95% Ethanol saturated with mercuric chloride (30%).

Time for staining - 5 minutes.

The strips were washed 4-6 times in tap water or 1% Acetic acid and finally as before in methanol, and air dried.

In order to improve separation of plasma proteins on paper the following experiment was performed.

A square (14 x 14 cms) of Whatmans No. 1 paper was taken and marked into quadrants lightly with pencil. Wicks of corresponding width and 17½ cms long were also prepared. After equilibration the plasma samples (control and haemolysed specimen from operation) were placed on the origin marked thus.



Electrophoresis was done in one direction then turned

round 90° and continued in the second direction. The following buffer was employed in this technique.

Buffer

8.8 g sodium borate decahydrate ($Na_2B_4O_7.10H_2O$) and 4.65 g boric acid (H_3BO_4) made up to 1 litre.

1st run: Overnight 17 hrs. 100 V 1.2 mA (total)

2nd run: Overday 7 hrs. 200 V 2.4 mA (total).

With this technique there is no need for equilibration before commencement of the second run as this has already been done, and the same buffer used for the second phase.

An improved modification was tried with this technique using two different buffers as follows, and the results are seen in Figs. 6.1 and 6.2.

Buffers

1st Run	1.84 g Barbitone 10.3 g Sodium barbiturate	1 L. u = 0.05
	2.76 g Barbitone 15.45 g Sodium barbiturate	1 L. u = 0.075

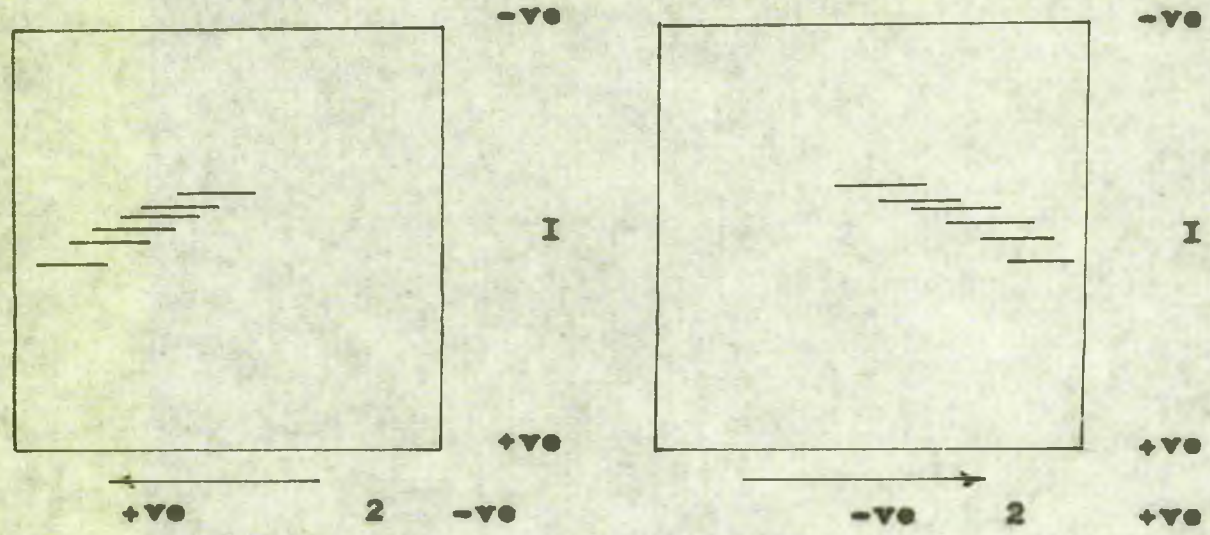
u = Ionic strength

The first run of lower ionic strength was done over 6-7 hrs. at 200 V and 4°C, (Fast run).

The second run of higher ionic strength was done overnight - 17 hrs. - at 100 V and 4°C, (Slow run).

The photographs although showing a distinct difference show up in even greater contrast in reality.

The order in which the runs are done and whether the polarity is reversed or not gives the following changes.



I μ = 0.075/100V/17 Hrs. I μ = 0.05/200V/6 Hrs.

2 μ = 0.05/200V/6 Hrs. 2 μ = 0.075/100V/17 Hrs.

The runs were dried and stained as mentioned above. Although paper electrophoretic separations may be scanned for protein density - it is - at its best only semi quantitative for the following reasons.

1) The staining reagents used are mixtures of dyes which are selectively staining different protein bands with differing intensity, i.e. dyes have different affinities for staining the same protein.

2) There is no linear relationship between quantity of protein present and the amount of dye staining the protein.

For these reasons quantitation is best left to the cellulose acetate technique.

High Voltage electrophoresis is unsatisfactory for protein separation partly due to the large heat production which causes denaturation, but probably more important the paper

2-way plasma protein separation using paper electrophoresis.

FIG. 6.1. (Control) →

FIG. 6.2. (Final) Haemolysed Plasma ↓

becomes too dry during the run - in other words equilibration is never reached, and separations are exceedingly poor.

Results

The results of plasma protein separation of pre and post perfusion specimens are seen in figs. 6.1 and 6.2. The characteristic primary bands are seen with unfortunately intermediate tailing bands which cannot be eliminated using paper for the separation.

The main differences between the control and final specimens are that the α_1, α_2, β and γ zones appear more concentrated in the latter. The same volume of plasma was used in both runs.

SECTION 6 B **Cellulose Acetate Electrophoresis** (CAM)

Many reviews exist on the use of cellulose acetate membrane for protein separations since KOHN introduced it in 1960.

The separation of plasma proteins was done using both

1) large Oxo - stripe 5 x 20 cms and

2) small Millipore strips 1 x 3 inches.

METHOD

1) The large CAM strips were cut into usually 2 pieces giving 5 x 10 cm sizes. Varying voltage, and running time the optimum conditions were found as follows.

Buffer	TRIS	78.65 g/L	1 Litre	pH 8.6
	EDTA	7.8 g/L		μ = 0.07
	Boric Acid	6.0 g/L		0.07M

Size of CAM = 10 x 5 cms.

Running Time 13 hrs.

Voltage 4.5 v/cm = 45 V.

Current 0.7 mA.

Prolonging running time to 16 hrs. gives bands which are less discrete. If two CAM's were run the current would be 1.4 mA approximately, i.e. doubled.

At a voltage of 355V and 4.9 mA condensation problems arose. Thus for all runs the above conditions were maintained and not exceeded. The same tank and power pack was used as with paper.

The cellulose acetate membrane technique

Great care must be exercised in handling these large CAM strips. It is a good idea to use small pieces of parafilm to wrap round the ends of forceps legs.

The membrane is gently floated on to the buffer and the latter allowed to permeate the underside first. Only then is the membrane completely submerged for a few minutes. This procedure if carefully done prevents air bubbles becoming entrapped in the membrane.

The membrane is removed from the buffer and gently blotted on filter paper. It should look completely homogeneous. If any white spots remain it is indicative of trapped air bubbles. This can be dealt with in two ways.

1) Discard

2) Leave overnight in buffer.

After blotting the membrane is placed in the electrophoresis tank, the wicks (paper or L.K.B. wicks) are placed on each end - 1/4" overlap and the lid replaced. The apparatus is equilibrated for 30-60 minutes. It is assumed that the electrode compartments have been filled level with buffer and that the wicks are buffer impregnated and gently blotted before being added to the chamber.

Application of sample

The special L.K.B. sample applicator was used to add 2.5 - 5.0 µl plasma to the membrane.

The sample was added, as in the paper technique, on a point 3/8ths of the way along the membrane from the cathode end. As an alkaline buffer was used the proteins migrate (with the exception of some globulins) towards the anode.

Occasionally bromophenol blue (dye) was added to the plasma sample as a marker. The dye migrates just ahead of the albumin fraction (fastest mobility). The dye may be added.

1) directly to the plasma prior to application or

2) the dye may be added at the origin as a very small drop.

Staining procedure

After each run the membrane is removed, placed on blotting paper and dried in an oven for 5-10 minutes. Alternatively it may be placed in an aqueous T.C.A. (trichloro-acetic acid) bath 5%. Both these procedures denature and fix and protein bands - thus avoiding diffusion after separation.

The membrane was then placed in the dye Ponceau Red (S) - 0.2% in 3% T.C.A. Care must be taken in floating the membrane on the dye surface to allow complete penetration before completely submerging it for 5 minutes.

The background dye (non protein areas) is eluted from the membrane by repeated washings in clean 5-10% acetic acid (aq.). When only the protein bands remain against a whitish background, the membrane is air dried and then kept flat between glass plates.

Electrophoretic separation of plasma proteins on cellulose acetate membrane (large strip)

FIG. 6.3.

The Melrose experiment final plasma.

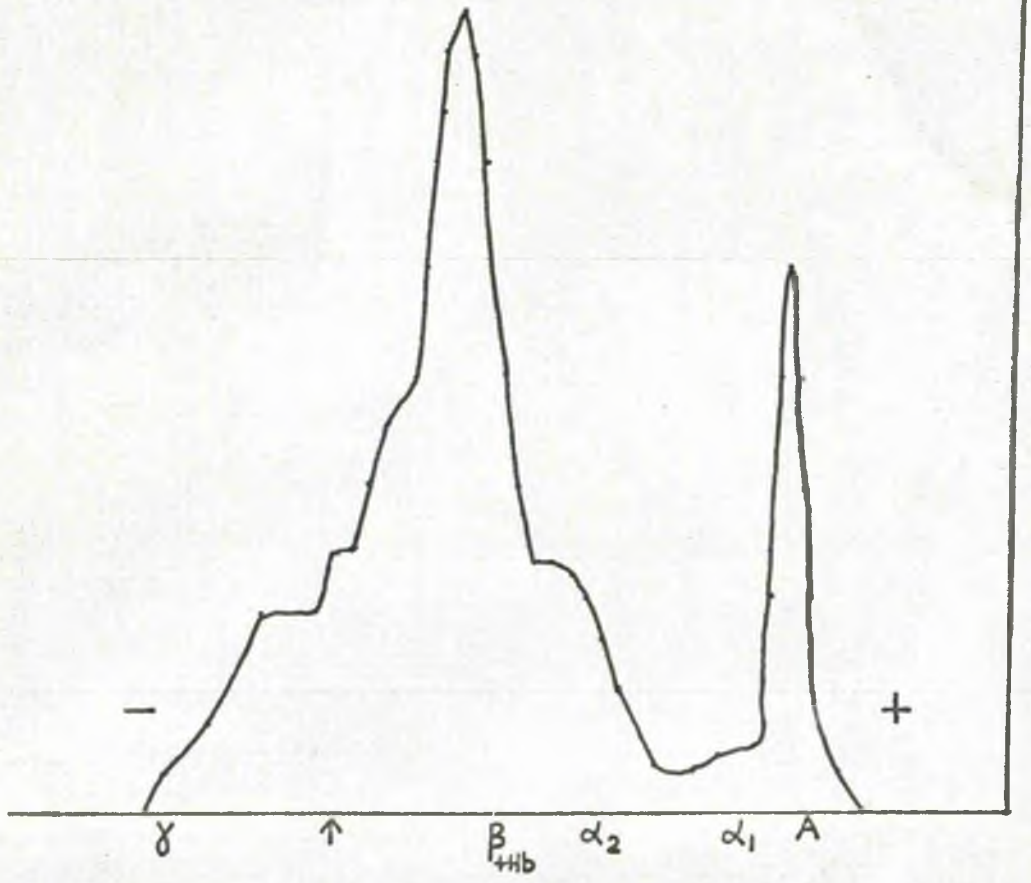


Fig 6.3 Scanner absorption
X4
Filter — ORI Deep Red.
↑ = application of sample

Alternatively - especially when the protein concentration is low, the dye Nigrosin may be used, 0.002% in 2% acetic acid. Since it is not absorbed linearly by protein as the concentration of the latter rises, it is better to use it only as a detector of low protein concentrations.

Aqueous solvents are preferable to alcoholic ones since the latter produces marked curling and shrinking of the cellulose acetate membrane unless passed through an aqueous bath finally.

Estimation of protein bands (qualitative and quantitative)

The estimation of protein bands may be done by

1) Rendering the membrane transparent by use of Whitmore White Oil 120 and assaying the protein in an E.E.L. Scanner. This is the light transmission method used here. Absorption is noted at 550 mμ or by using the appropriate filter. An integrator was not fitted to this instrument so that direct quantitative analysis was not easily obtained. The importance of the above oil is that it has the same refractive index as the membrane, which must be completely dry before it is placed in the oil. This method was used in Figs. 6.3 - 6.5 inclusive.

2) The individual bands may be eluted by using the original buffer or sodium hydroxide, after the bands have been cut out from the membrane. The colour intensity is then obtained in a spectrophotometer at 570 mμ. In this method it is essential that good separation is obtained. Using plasma proteins is difficult, since the larger electrophoresis is run the flatter and broader are the peaks.

Electrophoretic separation of plasma proteins on cellulose acetate membrane (large strip)

FIG. 6·4

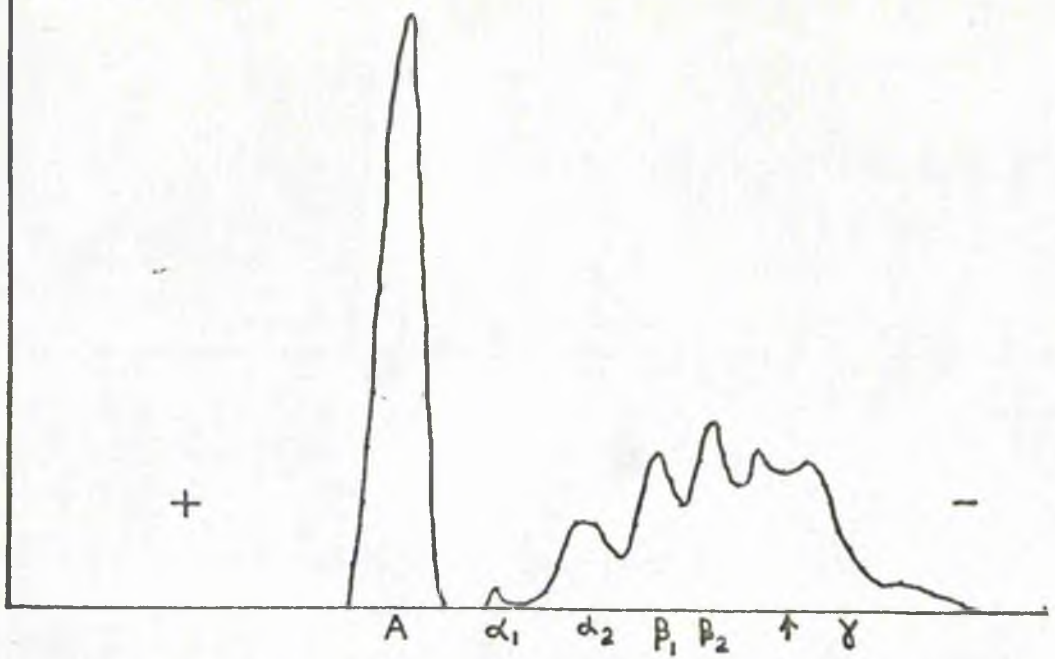


FIG. 6·4 Control plasma.
Pre perfusion.
Scanner absorption
x2
Filter - Blue and Green.

↑ = application of sample.

FIG. 6·5

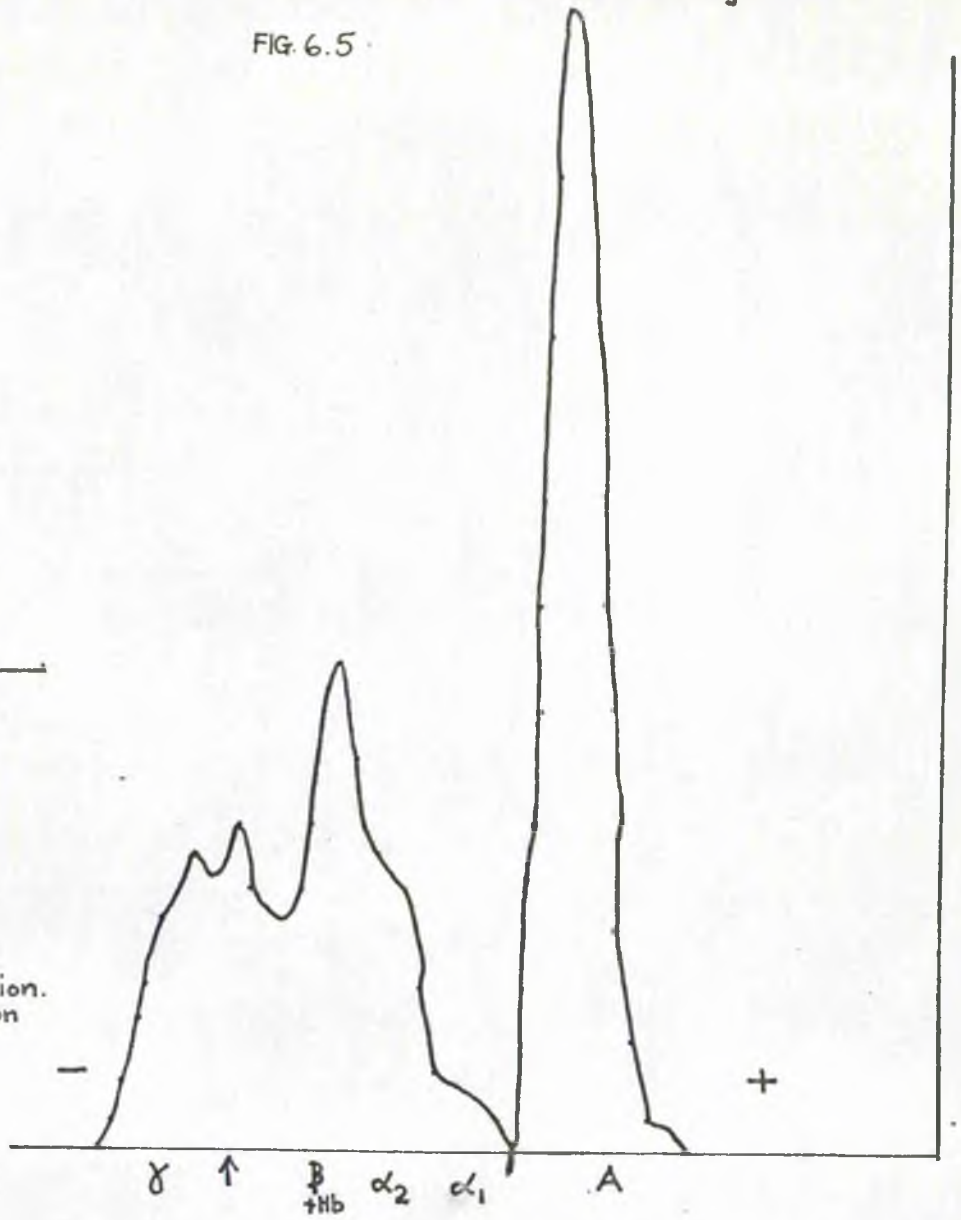


FIG. 6·5 Plasma
End of full perfusion.
Scanner absorption
x 3.
Filter - Blue

As has been pointed out elsewhere (acrylamide gel electrophoresis) one can obtain just as much value and information from electrophoretic separations by careful observation and photography, as can be obtained by calculating the areas of the individual peaks - as it done with the automatic integrator.

The Melrose experiment: Electrophoretic results : Fig. 6.3.

An 'in vitro' extracorporeal circulation, described in the introduction, was set up and blood circulated through the machine, heat exchanger, filter and twenty feet of silicone tubing for 6 hours.

Blood samples were taken at 45 minute intervals. The results here are from plasma aliquots taken at the beginning and end of this mock perfusion. Very distinct haemolysis took place which increased with "perfusion" time as can be seen in Fig. 5.5. The large increase in the "β globulin" band is due entirely to the free haemoglobin present in the plasma.

Operation: J.M. 12/67 : Figs. 6.4 and 6.5 This three hour perfusion gave similar results to those above.

Control = Plasma sample at commencement of full perfusion

Final = Plasma sample at end of full perfusion.

The haemolysis of the blood was not as severe as that seen in the Melrose experiment, due to the time of full perfusion being 50% of the Melrose duration. Hence it is seen that the globulin peak - although larger than normal is smaller than that seen in the Melrose "pseudo perfusion"

experiment.

Using different filters the intensity of the peaks are altered as shown.

The results of these separations will be discussed in detail at the end of this electrophoretic section. Suffice it to say the large increase in the "β globulin" peak is largely due to haemoglobin which migrates towards the anode at the same rate.

CELLULOSE ACETATE MEMBRANE II

Millipore apparatus

2) This involves the same techniques and care as the above method but on a smaller scale.

The opportunity arose to use a micro technique for plasma protein separation on CAM.

This had several advantages over the above method.

1) Very rapid separation - 20 minutes.
2) Even more discrete bands
3) Rapidity and reproducibility of results
4) Conservation of materials
5) Advantage of multiple runs in multiple individual cells connected to the module.

Buffer

Diethylbarbituric acid 0.331 gm.

Sodium diethylbarbiturate 1.848

this was made up to 120 mls. with water (gentle warming is necessary). The solution has a pH 8.6 and ionic strength

μ = 0.075.

Samples (0.3 ul) were added to the cell by a special dropper or applicator. The technique of equilibration of CAM with buffer is similar to the large scale method above.

Separation of plasma proteins gives best results with a voltage of 100 V and a 17 min run (more or less exactly), with these small CAM strips, heating is not carried out immediately after the run as protein precipitants are used with Ponceau Red to achieve the same effect.

Ponceau S	0.2%
T.C.A.	3.0%
Sulphosalicylic Acid	3.0% in water.

The CAM strip after staining was rinsed in 5% Acetic acid several times until the background was clear. It was then removed from the acid and gently blotted in preparation for densitometry.

Preparation for densitometry

1) The CAM slide was placed in 95% Ethanol for one minute.

2) After gentle blotting the slide was then immersed in the clearing solution (30% Ethyl acetate and 70% glacial acetic acid). Separation side up for one minute also. It is essential that this upper side is NOT touched.

Finally it was removed by forceps and left to dry - with NO BLOTTING - separation side up.

Integration of the separation procedure was not able to be done to instrument idiosyncrasies.

Fig. 6.6. Cellulose Acetate Membrane (II) Plasma protein electrophoresis: Control (right) and Haemolysed plasma (left).

The results of operation plasmas are seen in Fig. 6.6. The bands have become a little blurred due to enlargement from 1 x 3" (strip size).

DISCUSSION and CONCLUSION

1) The plasma protein changes that take place during perfusion appear minimal.

2) The 'apparent' increase in β globulin is due to concommitant migration of free haemoglobin.

3) Tailing of all fractions is more pronounced.

4) No new bands were observed.

SECTION 6C

Acrylamide gel electrophoresis

Acrylamide gel electrophoresis is another excellent media of recent innovation which gives similar results to those obtained with starch gels but has very distinct advantages.

1) Acrylamide gel electrophoresis is quicker

2) Technique is easier

3) Reproduction is more reliable

4) It can be used in a wider range of buffer than starch.

5) It can be made with differing pore sizes according to the type of protein(s) to be separated.

6) The gels after destaining are transparent in non protein areas and consequently densitometry and photography gives better results than starch and paper.

7) The protein bands are sharp and there is little evidence of failing.

Apparatus

1) Standard Shandon Acrylophor disc tank.

2) Volkmann Power pack

3) Destaining tank.

Method

Two methods were used 1) Discontinuous and 2) Continuous buffer systems. In the former a different buffer is used to form the gel and another for the electrode buffer, whereas in the continuous system the same buffer is used for both gel formation and electrode buffer.

The advantage of the continuous system is that equilibration of the tank constituents may be allowed to take place for ½-1 hour and also any impurities are removed from the gel before the plasma sample is layered under the top buffer (current switched off) on to the upper surface of the gel.

A 7.5% acrylamide gel was used as this gives good separation of protein molecules in the M.W. range 400,000 - 10,000, which includes all the major plasma proteins.

CHEMISTRY

The acrylamide gel was prepared by mixing acrylamide monomer with the cross linking agent BIS and N,N,N,'N' tetramethyl ethylene diamine (TEMED), and ammonium persulphate as catalyst accelerator. Potassium ferricyanide was used to prevent the gel formation proceeding too rapidly.

THE DISCONTINUOUS TECHNIQUE P.H.P.G.

Three solutions were prepared

SOLN I

4.8 mls. conc Hydrochloric acid (Analar)

0.46 mls. TEMED (N,N,N,N, tetramethylethylenediamine

36.3 gm TRIS (hydroxymethyl-amino-methane) made up to 100 mls. with distilled water.

SOLN II

30 gms. Acrylamide

0.8 gms BIS (N.N methylene bis acrylamide

0.015 gms potassium ferricyanide this is dissolved in water (distilled) and made up to 100 mls.

SOLN III

0.625 gm Ammonium persulphate made up to 250 mls. with distilled water.

To make the gel for the discontinuous technique solutions I, II and III were mixed with water in a 5:10 : 20:5 ml. ratios.

This finally gives a gel of 7% Acrylamide in 0.375 M Tris HCl.

The Electrode Buffer for Discontinuous technique

6 gms TRIS

28.8 gms glycine mixed with water and made up to 1L.

This gives a 0.05 M Tris glycine buffer, pH 8.6

To the disc tubes (0.5x8 cms) stuck in the plasticine holder is added 1 ml. of the gel solution (from syringe) which is allowed to set - 10-30 minutes - according to catalyst and accelerator concentration employed. A little distilled water was added to the top of each tube.

The lower anode chamber was filled with the electrode buffer - enough to cover the anode, and the tubes when set are placed in the holder (maximum 8 tubes), and placed on top of the anode compartment. As alkaline buffers are being used the cathode is at the top of the electrophoresis tank.

The cathode - top compartment is now placed on the apparatus and filled with electrode buffer also.

As in column chromatography the plasma sample was made denser than the buffer by adding 5-10% sucrose. If denaturation of blood samples from cardiac surgical patients were not being investigated, urea could be added in lieu of sucrose.

Plasma sample

All plasma samples were stored at 1-3°C and were used as soon as possible after collection. 0.1 mls. plasma was mixed with 0.1 mls. 20% sucrose and mixed gently but thoroughly. 10 µl of this was added to each gel tube which is equivalent to 5 µl. plasma.

If the concentration of protein present was 8 gms/100 mls. this is equivalent to a concentration of 200 µgms per tube.

Similarly if the plasma protein concentration was 6 gms/100 mls. this is correspondingly equivalent to 150 ugms/tube.

Usually the total protein concentration varied between 4-6 gms/100 mls.

The protein concentrations shown on the viscosity table (Section 4) were obtained by the following methods:

1) Nitrogen determination

2) Biuret estimation

3) Ultra Violet absorption at varying wave lengths.

Application of plasma sample

5 µl of plasma was carefully layered on top of each gel tube underneath the upper cathode electrode buffer. As this is the discontinuous technique, an equilibration or pre run must NOT be done prior to the application of the plasma sample.

For 10 minutes the current was left at about 1 mA per tube. After the protein had entered the tube the current was increased to 4 mA/tube. This method uses constant current. The voltage in this method commences with 30 V and rises to near 60 V.

The separation lasted 30 minutes. The current was then switched off. The gels were removed from the tubes by inserting the needle of a syringe (full of distilled water 1 ml.) down the side of the gel - i.e. between the gel and the glass side. This loosens the gels which were immediately

stained in Amino Black 10 B (1% in 7% aqueous acetic acid) for 75 minutes, to stain the protein bands.

Destaining This may be accomplished as described in former electrophoretic methods - by simple elution with dilute acetic acid (1-2%) or it can be done more quickly electrolytically, as described below.

The gels were washed gently with distilled water after staining and then carefully placed in a perspex holder. This was placed in the destaining tank. Dilute acetic acid 1-2% was added to the tank and the lid containing the electrodes placed on top. The current was switched on and kept around 200-225 mA for 1-1½ hours.

This procedure elutes the dye from non protein areas of the gel. The acetic acid may be changed for fresh electrolyte until the non protein areas retain no further dye.

Evaluation of separation

As mentioned before, although densitometry can be done - there is no true linear relationship between protein concentration and so called quantitative estimation, especially at higher concentrations of protein. Therefore evaluation of these separations was done by simple observation and photography.

II Continuous Buffer Technique

The advantage of this technique is the homeostasis of both electric field and buffer ion environment during the electrophoresis.

A T.E.B. buffer was used.

29.0 gms TRIS

2.5 gms. Na_2 E.D.T.A. (disodium ethylene diamine tetracetic acid)

0.95 gms Boric Acid.

This was made up to 2 .6 litres and gives a buffer solution of pH 9.2 and 0.08 M.

The solutions I, II and III used in the discontinuous technique were used here, and the gel made up thus :

Ratios for gel formation Soln I : II : III : Buffer are 1 : 2 : 4 : 1 respectively.

The technique used here was the same as with the discontinuous (T.M.T.G.) technique except that after the gel had been formed and the buffer added to both anode and cathode compartments, the current was switched on for 30-60 minutes.

After this time the current was switched off while the plasma samples were added to the upper surface of each gel.

The results of pre and post full perfusion plasmas using both the Discontinuous (T.M.T.G.) and Continuous (T.E.B.) buffer systems are seen in Fig. 6.7 - in the middle of which is a fresh control plasma (i.e. not stored).

Starch Gel Method SECTION 6 D.

Sometime was spent on preparing Fisons soluble starch for electrophoretic work. Using this particular brand

of starch best results were obtained using 13 gms and 152-154 mls. buffer, this is fairly critical for good results.

It is very important what type and brand of starch is used, i.e. whether hydrolysed or not.

The buffer used was

Boric acid 0.133 g/100 mls. = 0.0215 M

and Sodium hydroxide 0.0344 g/100 mls. = 0.0086 M

The two solids were added together and the volume made up to 100 mls.

To this was added the starch. The mixture, in a conical vaccum flask was heated with constant swirling over a naked bunsen flame, until the suspension becomes a viscous opaque mass.

After thorough but brief de-bubbling by vacuum (1 min.) the gel was poured into the small perspex trays.

This work was unable to be continued because the trays available were too narrow, short and shallow to permit adequate slab formation.

The results obtained with paper, cellulose acetate and polyacrylamide gel however made the use of starch gel superfluous.

Discussion.

Good separations of the individual plasma proteins were obtained on paper, cellulose acetate and polyacrylamide gel. On paper just the basic separation into Albumin,

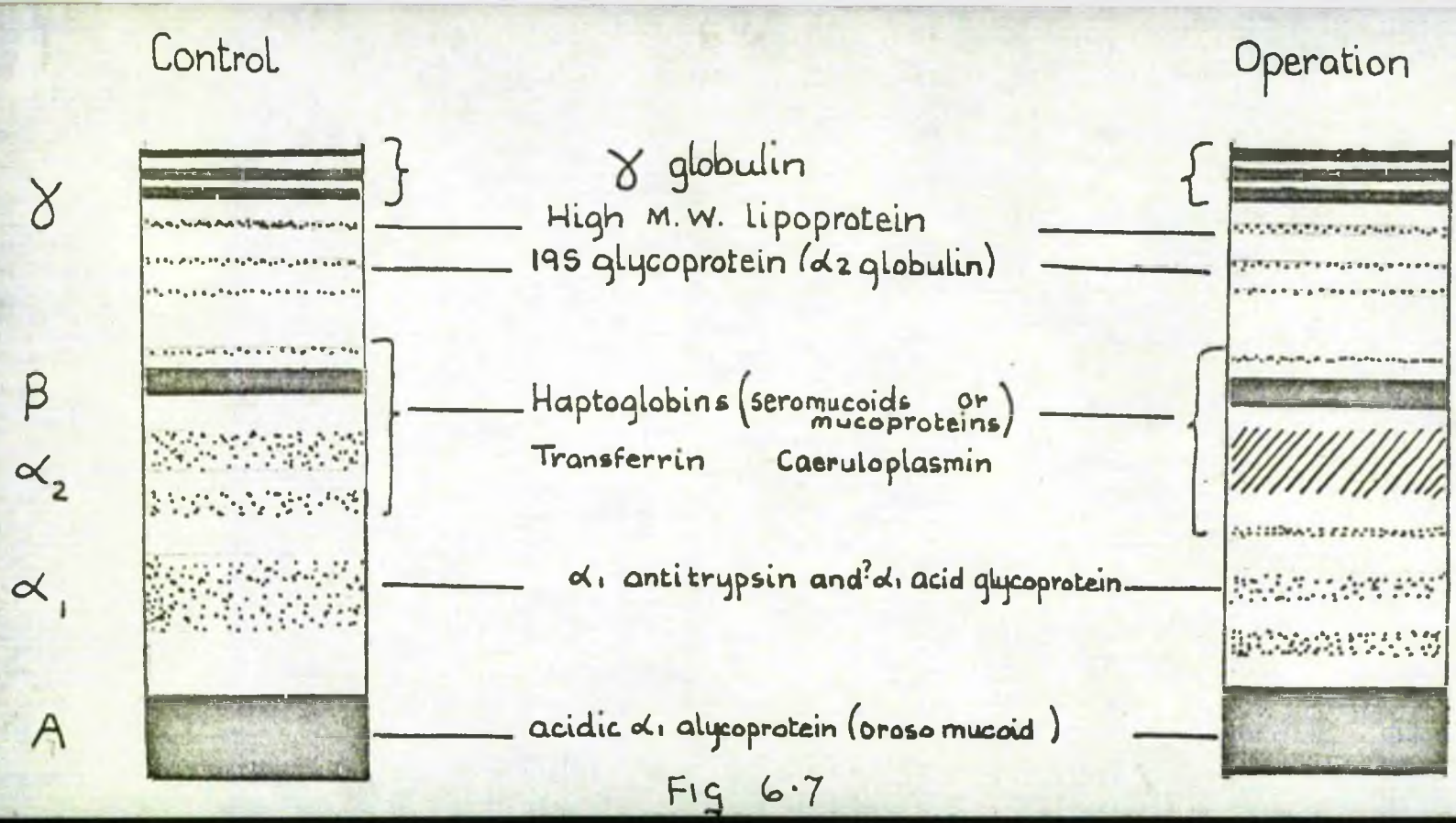
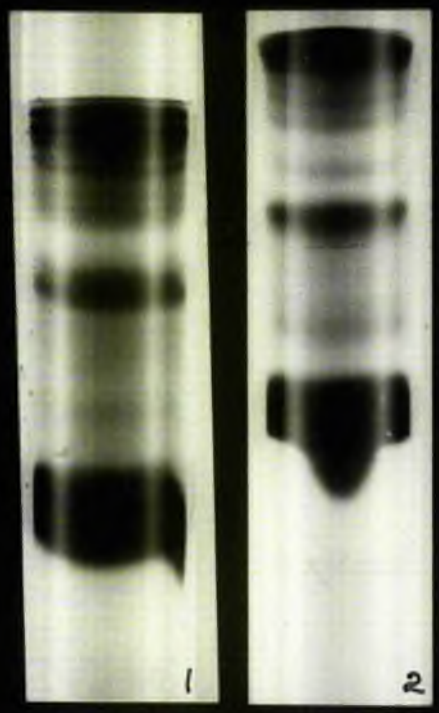
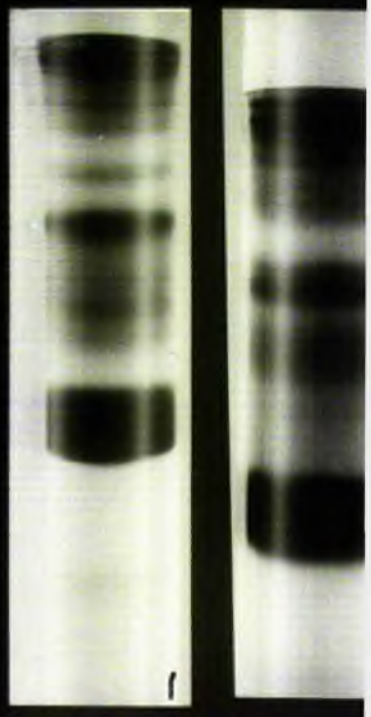


Fig 6·7





1) Plasma Control using T.E.B. Buffer.

2) Plasma Control using T.H.T.G. Buffer.

Fresh non stored plasma (for comparison) →

FINAL PLASMA (Haemol

1) T.E.B. Buffer (Continuous)

and

T.H.T.G. Buffer (2) (Discontinuous).

α_1, α_2, β and γ globulins are seen. However since haemolysis of some extent has occurred in samples of blood taken at the end of perfusion, haemoglobin merges with the globulin and thus give abnormal results when the peaks are quantitatively scanned.

During full perfusion the plasma proteins remained remarkably constant over ½ - 2½ hrs. It is thus possible to compare the fractionation results taken at half-hourly intervals.

The following conclusions may be derived from simple electrophoresis and quantitative scanning.

1) There is virtually no visible change in concentration of α_1 and α_2 globulins.

2) β globulin showed minimal changes which showed no relationship with the changes in total protein concentration.

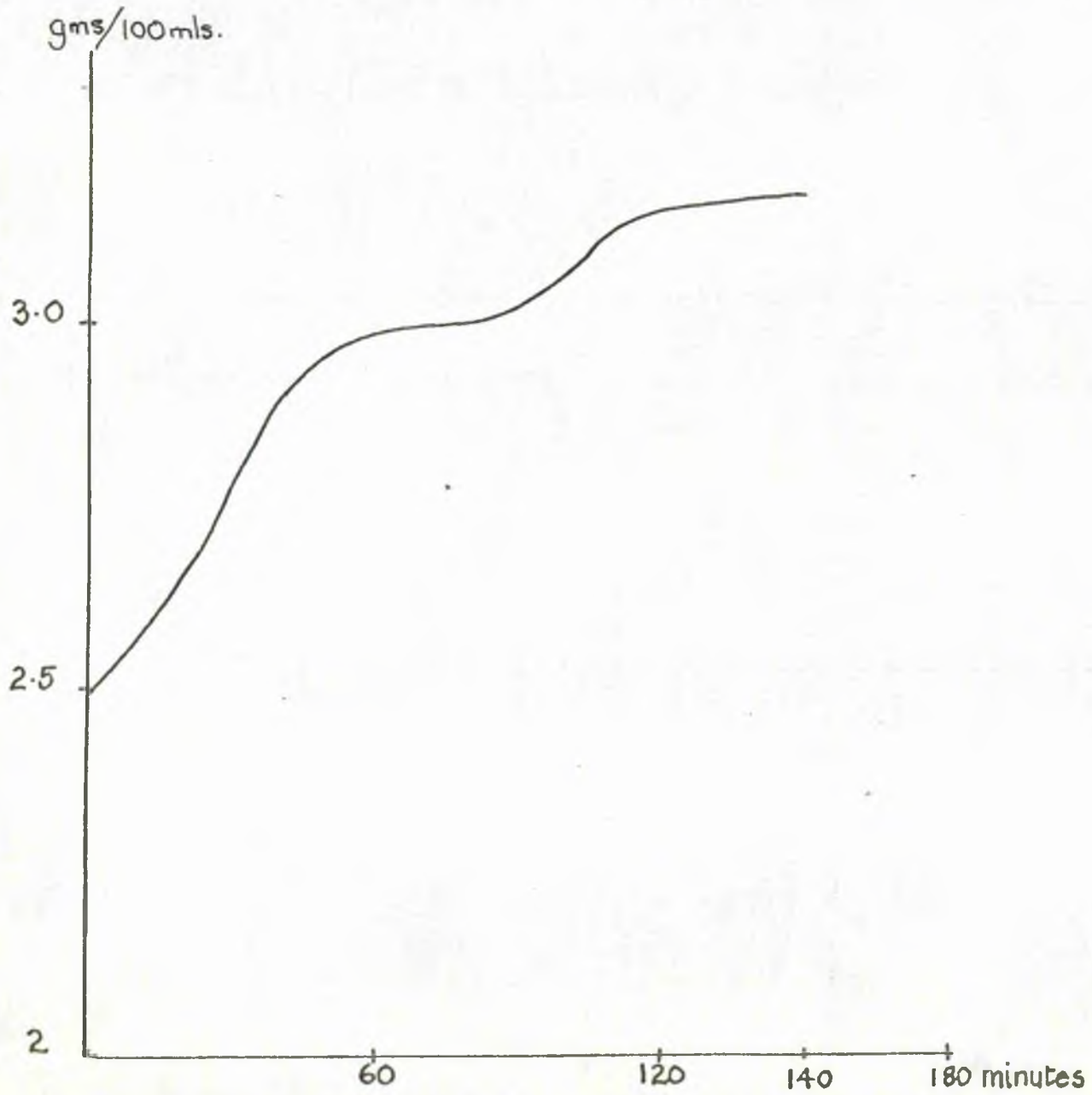
γ globulin showed small but distinct changes which bore no relationship with neither cases nor the total protein in the particular specimen. It is interesting that stored blood shows separate γ globulin bands which are not seen in fresh plasma, see Fig. 6.7.

Albumin changes mimicked those of total protein changes indicating that the former was more or less solely responsible for changes in the latter. This is mainly due to 2 factors.

Change in serum albumen during full perfusions of varying duration.

FIG. 6·8

(mean of 8 cases)



1) Albumin having the smallest molecular weight and greatest osmotic effect in the blood obviously has a profound effect on haemodilution or haemoconcentration under normal conditions; this fact is enormously important under the conditions of surgery where blood loss and blood gain (transfusions) are proceeding side by side but not proportionately.

2) Albumin is present in plasma to the extent of 54-58%. In most cases (90-95%) ½ - 2½ hrs. there was a slight rise in both total plasma protein and albumin during perfusion. Most of these cases had virtually no blood added during perfusion and blood loss was minimal. Obviously there is some loss of body fluid at the operation site and this could conceivably be responsible for an "apparent" homeostatic haemoconcentration during full perfusion.

It is possible that some of the changes which start taking place during perfusion continue for some time in the post operative period. In this respect it is worthy of note that α, acid glycoprotein, α_1 antitrypsin, haptoglobin, caeruloplasmin and $_2$ globulin increase post operatively and do not return to normal until the 18th post op. day. This is a response to trauma and is part of the acute phase reaction, Werner, 1969.

We have seen that in perfusions with no simultaneous blood transfusions or saline/dextrose drips in use the albumin rises slightly, Fig. 6.8.

Total perfusions time		Change in albumin gms/100 ml.			
H.D.	45 min.	2.4 ⟶	2.9	= 0.5 g	Gain
D.S.	55 min.	2.5 "	3.0	= 0.5 g	"
M	165 min.	2.9 "	3.6	= 0.7 g	"
D	165 min.	2.6 "	3.0(3.1)	= 0.4(0.5)	"
A.C.	165 min.	2.9 "	2.4	= 0.5 g	Loss

As can be seen from this table the rise in albumin concentration is remarkably constant and bears no relation to the total perfusion time and follows a sigmoidal curve. The maximum increase being reached within 45 minutes (this is approximately the shortest full perfusion time).

A.C. 165 mins. was one of the largest full perfusion times and due to its length, extra blood had to be given, also there was a good urine flow in this case, thus affecting the labile blood volume. These two factors are undoubtedly the main cause of the decrease in serum albumin seen in this case.

Paradoxically serum albumin, transferrin, α_1 and β lipoprotein and-pari passu- total protein decrease following operations. This is a transient fall (WERNER 1969) and returns to normal around the 9th day.

The total protein post operatively falls 9% from 6.83 gm to 6.44 gm. (3rd day) and then rises to original level. Sterling 1965 has shown that the fall of serum albumin is the main cause of fall of plasma proteins due to extravasation loss in the wound site. Mouridsen 1967 has studied the turnover of albumin before and after operations and Jarnum, 1961 has shown that plasma protein exudation takes place in the peritoneal cavity during laparotomy.

It is of interest that γ globulin and α_2 macroglobulin which appear to remain relatively unchanged during cardio-pulmonary bypass remain unchanged post operatively with the following proviso, that γ globulin rises only when post operative complications occur, e.g. infections such as pneumonia.

The fact that α_2 macroglobulin remains constant post operatively (WERNER) excludes significant haemodilution or blood loss during this period.

In the photographs of Fig. 6.7 plasma protein separation on acrylamide gel, samples have been taken from the beginning of perfusion (control) and end of perfusion (final).

Thirteen bands may be discerned which compare very favourably with the separation of Poulik and Smithies, 1958 using a combination of starch gel and paper.

The fine normal bands found on paper electrophoresis resolve into twenty two zones (2-way) or seventeen zones in one way separations.

Plasma protein separation in the Control sample from commencement of full perfusion

Prealbumin. This normally occurs as a pre-tailing peak of albumin. In this separation there is a faint ill defined band running someway in front of the albumin band. This is present on all samples and could not be anything else but prealbumin.

Albumin This peak may well conceal the acidic α_1 glycoprotein (orosomucoid).

α_1 globulin consists mainly of α_1 antitrypsin and α_1 acid glycoprotein. One or two haptoglobins may be present in this band as well.

α_2 globulin consists mainly of haptoglobins, of which there are roughly six.

The latter partially merge in with the classical β globulin which also contains transferrin and caeruloplasmin.

Between β and γ globulins, the 19 S glycoprotein, heat labile glycoprotein and $\beta\alpha_2$ globulin are found.

The high molecular weight lipoprotein - which is hidden in one way electrophoresis - is probably obscured by the globulin bands.

Haemolysed plasma from the end of full perfusion

The main difference appears in the α_2 and β globulin bands, mainly due to haemoglobin. It would appear however

that because the albumin, α_1 and γ globulins are identical with the control than the α_2 and β globulins would most likely show no difference at all - if the haemoglobin could be removed. This additional fractionation was tried later using a column chromatographic techniqus.

Since no difference can be seen in the separation of the γ globulin fractions with the control it would appear unlikely that there is any large change in the lipoprotein fractions. However sub fractionation of plasma proteins is needed to prove this point.

Control fresh plasma centre of Fig. 6.7.

This shows absence of individual γ globulin bands which are seen in both control and final perfusion plasmas. Presumably these changes must be explained by the fact that perfusion blood has been stored for 1-3 days at 2-3°C and therefore cryogenic in origin. There appears to be little difference in separation from the operation specimens of the other protein bands.

CONCLUSION

1) Best fractionation of plasma proteins were obtained using acrylamide gel, 13 bands were seen.
2) Stored blood - used in perfusions showed separate γ globulin bands not seen in fresh plasma.
3) Minimal changes are seen in the albumin, α_1, α_2, and γ globulins during perfusion.
4) The increase in "β globulin" is due to similar migration of the free haemoglobin.

SECTION 7

Ultracentrifugal studies of plasma proteins

Introduction

Following electrophoretic investigation of plasma proteins from cardiac operations, the opportunity was taken of investigating the changes, if any, of the same plasma samples on the ultra centrifuge. Mention has been made of the factors that enable electrophoretic separation of plasma proteins to be obtained.

Ultracentrifugal analysis of proteins relates directly to density, size and shape. Thus the results from both these methods do show some dissimilarities as well as similarities. Where differences do occur in respect of a certain protein however the two measurements help to characterise the plasma proteins together with functional and chemical properties.

In this work the sedimentation coefficients, S_{20} of plasma proteins have been studied. 10% plasma was mixed with isotonic saline (0.9%) - a medium of lower specific gravity - and by centrifugation sedimented towards the periphery of the rotor cell under the influence of the strong gravitational field. The rate of sedimentation is characteristic of the protein - or in this case a group of proteins - present. This value is the sedimentation coefficient.

Schachmann, 1959 has reviewed the use of the ultracentrifuge in biochemistry in a recent monograph.

The molecular weights of proteins may be studied by both sedimentation equilibrium and sedimentation velocity. The former gives the molecular weight directly in contrast to the velocity method, which although it is more rapid, depends not only on the density, size and shape of the molecule(s) but also on the diffusion constant and partial specific volume of the protein in order for the molecular weight to be calculated. The sedimentation equilibrium method has the disadvantage of being time consuming and requiring the necessity of constant control of centrifugation.

The sedimentation velocity method (used here) enables the sedimentation rate of the plasma proteins to be determined. The sedimentation coefficient (S) is defined as the velocity of the sedimenting molecules per unit of gravitational field. From the photographs seen in this section the sedimentation velocity could be calculated by measuring the movement of the peak with respect to time. The centrifugal field is easily calculated from the speed of the run (59,780 R.P.M.).

Using factors of temperature and viscosity the calculation of S in "svedbergs" may be obtained. 1 svedberg =

1×10^{-13} cm/sec/dyne/gm. Since S is a constant, (in a given solvent system) it may be used directly to compare differing molecular species. In this section the S values obtained from the peaks are compared with S values of known characterised plasma proteins.

Method

Following total protein estimation (total nitrogen, Biuret, and/or U.V. absorption) a small aliquot of plasma was taken e.g. 1 ml. and diluted to 10 mls. by gently adding isotonic saline (0.9%). 1 ml. of this was used for the ultracentrifuge run.

Haemolysed plasma, initially gave rise to some difficulty. After 10% dilution as above, only one peak could be obtained instead of the usual three.

It was apparent that either 1) the solution was too concentrated or 2) a change of wavelength of the solution may solve the problem. The latter appeared to be the better alternative.

Plasma protein concentration Most samples of plasma from operations gave total protein concentrations lower than normal. This was mainly due to the preliminary dilution by dextrose saline during the priming of the extra corporeal circulation.

Most total plasma protein concentrations varied between 4-5 gms/100 mls. These values after theoretical

dilution give values of 40-50 mgms protein/10 mls.

Further dilution was much to be avoided due to the possibility of denaturation and loss of existing peaks. It was therefore considered worth while changing the maximal absorption of the haemoglobin present in haemolysed plasma by converting the haemoglobin to carboxy-haemoglobin.

	Max. absorption mμ.
Haemoglobin	565
Carboxyhaemoglobin	535 and 571.

Accordingly coal gas was gently bubbled through the diluted plasma for 45-60 minutes and the ultracentrifugal analysis carried out immediately afterwards.

It would appear that the reason why only one peak (4.5 S) appeared initially was due to the orthochromatic plates of the ultra centrifuge being insensitive to red and thus in the haemolysed plasma the 7 S and 18 S peaks were unable to be obtained. It appears fortuitous that even the 4.5 S peak occurred in the oxyhaemoglobin haemolysed plasma specimen.

A normal unhaemolysed plasma was used as a control. No difference was observed.

As has been pointed out in the section on Haemolysis, the degree of free haemoglobin in plasma in the final specimens is of the order of 200 - 600 mgms/100 mls.

So 1 ml. plasma diluted to 10 mls. for the ultra-centrifuge analysis would contain haemoglobin of the order 2.0 - 6.0 mgms/10 mls.

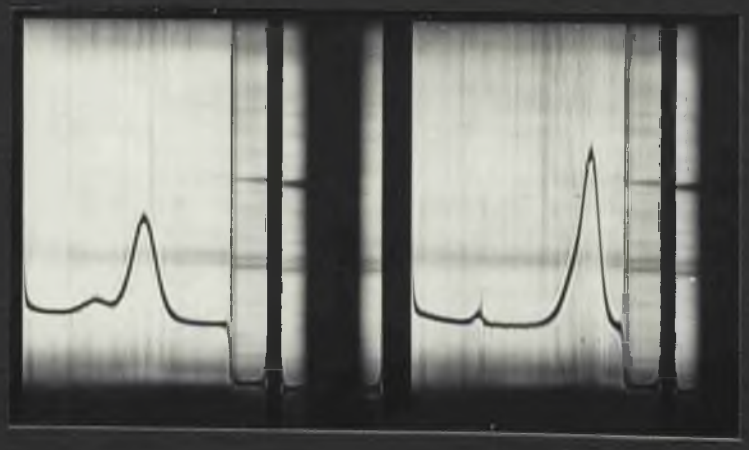


Fig. 7.1. Control plasma.

After the diluted plasma sample had been placed in the ultracentrifuge the latter was run up to a speed of 59,780 revs/min. and maintained for 1½ - 2 hrs. Serial photographs were taken during this process.

Results Figs. 7.1

Control Plasma

Three peaks were found

Mean 15.06 x 10^{-13} (3)

6.2 " (6)

4.04 " (9)

Haemolysed Plasma (final) from operation

Three peaks only still found.

Hb as Oxyhaemoglobin (A3)	Hb as Carboxy Hb (A1)	Hb as Carboxy Hb (A2)
1) -	17.01 x 10^{-13} (3)	17.45 x 10^{-13} (2)
2) -	6.08 x 10^{-13} (6)	5.3 x 10^{-13} (3)
3) 4.05x 10^{-13} (7)	4.23 x 10^{-13} (3)	3.99x 10^{-13} (9)

The figures in brackets indicate number of readings per peak, over the 1½ - 2 hour run.

Plasma protein concentrations

Control 4.4 gms/100 mls. plasma

A... 5.4 gms/100 mls. "

A.C. 4.1 gms/100 mls. "

Fig. 7.2. Haemolysed plasma (Final) R.M.

DISCUSSION

In normal plasma three main peaks are found with an S_{20} value near 4.5 S,

7 S, and

18 S

These figures are taken from results of normal plasma protein concentration i.e. 6-8 gms/100 mls.

As can be seen with the results above both the control and final (haemolysed) figures agree very closely, bearing in mind the protein concentration due to dilution at operation.

The results are seen in figures 7.1, 2 and 3.

No additional peaks are seen and the S values found of the peaks present indicate no significant fragmentation of the protein molecules during perfusion.

Only by fractionation of plasma proteins can the S values of the more minor components be obtained.

The three peaks mentioned above consist of the following moieties each of which may have one or more S values. Hence there is considerable overlap.

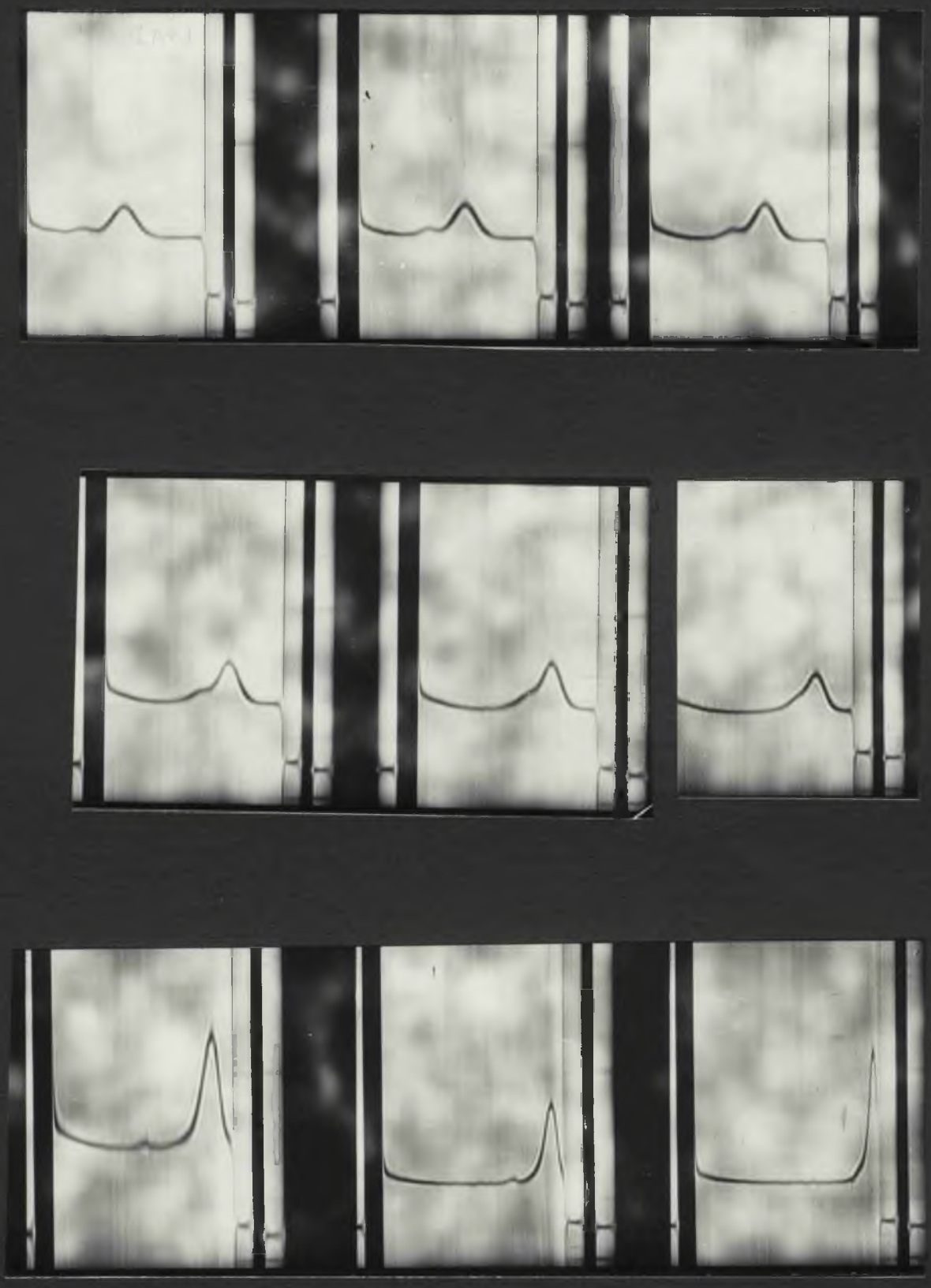


Fig. 7.3. Haemolysed plasma (Final) A.C.

		S_{20} values
	Pre-albumin	4.1
	Albumin	4.4 (4.0 - 4.6)
α_1	seromucoid	2.9 (2.4 - 3.5)
α_2	globulin	2.9
β	globulin	5.0
β	glycoprotein	
α_1	lipoprotein	
	transferrin	
β_1	lipoprotein	7.0
β_2	globulin	
	lipid poor euglobulin	
γ	globulin (6.6)	
α_2	glycoprotein	9.0
	lipid poor euglobulin	20.0

Small 12 S fractions have been observed which are associated with α_2, β and γ electrophoretic zones.

The γ zone as well as containing the 19S fraction is also associated with 28S and 44S components.

The 3S α_1 globulin is probably an acid glycoprotein.

The 4S α_2 globulins contain haptoglobin, caeruloplasmin and many enzymes.

The 5S β globulins are mainly iron binding proteins.

The 7S γ globulins possess acquired antibodies.

The 19S α_2 globulins are glycoproteins and the 19 S globulins are macroglobulins.

The latter contribute to the α_2 electrophoretic fraction as well as the γ fraction.

From ultracentrifuge work of the haptoglobins it is apparent that they migrate in the α_2 globulin zone of electrophoresis.

Therefore :

The 3 S fraction contains	α_1	globulin.	
4 S " "		albumin	90%
	α_2	globulin	10%
5 S " "	β	globulin	
7 S " "	γ	globulin	68%
	β	"	23%
	α_2	"	9%
19 S fraction contains	γ	globulins	33%
	α_2	"	67%

Clearly in the 3 peaks mentioned earlier on (Control) the 4.04 S contains Prealbumin

Albumin

α_1 globulins

α_2 globulins

?β globulins

The 6.39 S contains β globulins

γ "

α_2 "

and the 16.06 S contains γ globulins and

α_2 globulins.

<u>Conclusion</u> There is no overt change in the constituent proteins of plasma during full perfusion lasting up to 3 hours.

It will be appreciated that each of the three peaks obtained from ultracentrifugal studies of plasma proteins contains several proteins, some of which occur in two or more peaks. Nevertheless any change in the molecular weight of a protein or proteins during perfusion would alter the ratios of the S values characteristic of the individual peaks.

It is clearly seen from the above work that although ultracentrifugal and electrophoretic studies show many similarities there are a vast number of complicating factors e.g. a protein of one S value occurring in three electrophoretic

bands, and several different proteins having the same S value.

This clearly shows the different basic factors upon which the principles of ultracentrifugation and electrophoresis are founded.

Electrophoresis depends mainly on electric charge and, to a lesser extent - size of the protein molecule - depending on the media used.

Ultracentrifugation depends solely on shape, size, density, viscosity, partial specific volume and diffusion constant of the protein molecule. The latter three are required for the calculation of the molecular weights.

SECTION 8

Column Chromatography of plasma

Introduction

One of the major problems in the study of plasma proteins is the difficulty of achieving efficient separation for further characterisation. This has already been referred to in the Electrophoresis and Ultracentrifuge sections.

One aspect in which many of the plasma proteins differ from each other is that of Molecular weight e.g.

Albumin	70,000
Globulins	160,000
Fibrinogen	400,000
β lipoproteins	1,300,000

With the introduction of Sephadex Gels it has been possible to separate proteins of differing size. Accordingly plasma from cardiac operations was applied to columns containing G 200 of two different lengths. G 200 was chosen since this gel is less extensively cross linked than G 25, 50, 75 and 100. The approximate molecular weight exclusion limit is 200,000.

The theory of gel filtration is well known and documented and will be largely excluded from this work.

It was the initial intention of using G 200 in an initial separation of plasma proteins, concentrating the fractions and refractionating on the anion exchanger DEAE - Sephadex. However for reasons that will be explained, this latter separation was unable to be attempted although the DEAE column had already been packed.

Initially a column containing 3 gms of G 200 was used but was later substituted for one containing 7 gms/G 200. The theory of this change was to improve the resolution of the fractionation but against this was the fact that the longer (larger) the column - especially of G 200 - the longer elution takes. In fact several days were required for this. The object of comparing control with final plasma specimens from operations was to determine if any significant change had taken place during perfusion. Obviously it was of paramount importance to ensure that denaturation on the G 200 column due to prolonged elution, was kept to a bare minimum. Exacerbating this factor is the point that proteins in increasingly dilute solutions are increasingly more liable to denaturation.

The optimal criteria in this separation therefore was to obtain efficient separation of plasma proteins in the minimal time.

As a result of changes found in the electrophoretic techniques, these column chromatographic separations were

used to try to separate haemoglobin in plasma (haemolysed) from the true plasma proteins, which hitherto had been difficult to accomplish. This was mainly because of two factors.

1) Similarity of net charge and

2) " of molecular size

Albumin and haemoglobin having virtually the same molecular weight but differing more in shape. Haemoglobin may however dissociate into half molecules. Albumen however is more asymmetric with an axial ratio of 4:1 compared with 1:1 for Haemoglobin.

Since separation of proteins on G 200 is rather time consuming, more emphasis was laid on obtaining the optimum conditions for plasma proteins separations rather than the full elucidation of each peak. Since some of these peaks were broad it rather signified that further primary separation was desirable and this was obtained by

1) Increasing column length and thus V_t, and

2) Decreasing plasma protein application volume.

Whatever Sephadex Gel is chosen for protein separation it is necessarily a compromise between giving good fractionation of some proteins and poor fractionation of others.

Method Preparation of Gel:

G 200 of all Sephadex gels takes the longest time to

swell in water or buffer. This takes 2-3 days unless a strong urea (5-6 M) solution is used instead to obtain more rapid equilibrium. If urea is used it can easily be eluted from the column during packing with either water or eventual solvent.

A definite weight of G 200 was left to equilibrate with water, 6 M urea and Tris HCl/NaCl buffer on three different occasions. The gel in plenty of supernatant was left to settle and the excess volume gently decanted off. More buffer or water was added, the contents swirled and this action repeated. This was repeated a third time. The object of this was to wash away any fine particles present in the gel which would tend to "clog up" the column when it was being packed.

Following this procedure the gel and buffer in a vacuum conical flask was deaerated for at least 30 minutes and preferably longer. A column was chosen of minimal dead space at its lower end (outlet).

The size was 2 x 80 cms. and it had a water jacket. A circle of filter paper No. 4 or 540 was floated down the column containing buffer so it just covered the entire upper surface of the glass filter at the lower end of the column.

A thick slurry of the gel was gently and continuously poured down the column until it was full. The outlet was

kept closed for several hours while the gel was packing at the bottom of the column. Fresh gel was then pumped in at the top of the column from the conical flask and the outlet partly opened so that the level at the top of the column was kept constant. After 4-6 hours the outlet tap was opened fully and the column allowed to pack at its own rate. It became increasingly obvious that under no circumstances must packing be forced by pumping in gel in a "closed system" at the top of the column.

When the gel level had equilibrated near the top of the column, buffer was pumped in at the same rate as the effluent and the column left to finally equilibrate for several days. Care was taken to ensure that the column was absolutely vertical and the packing proceeded in an ascending level manner.

Determination of Void Volume.

The total volume of a Sephadex column V is expressed by

$$V = V_o + V_i + V_g$$

where V_o = water outside the gel particles.

V_i = water inside the gel particles

V_g = Volume of gel matrix.

The total volume of the large column was 220 mls.

FIG. 8·1 Fractionation of Plasma control . G.200

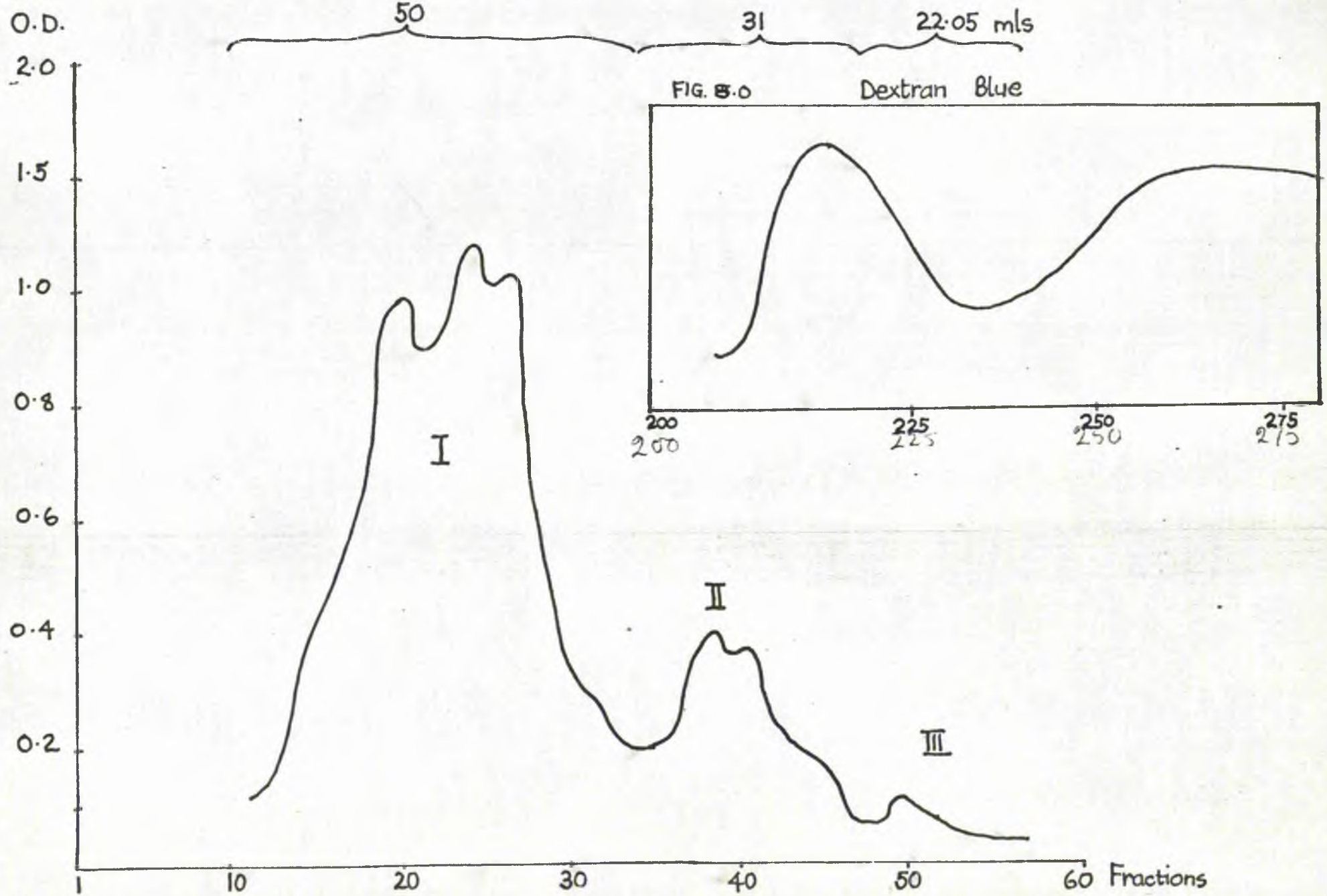


FIG. 8·0 Dextran Blue

V_i = Dry weight of gel x the water regain

$\therefore V_i = 6.7 \times 24 = 160.8$

$V_g = 3.2$ mls.

$\therefore 220 = V_o + 160.8 + 3.2$ mls.

$\therefore V_o = 56$ mls.

Dextran blue was used to determine the void volume accurately. 2 mls. of a fairly concentrated dextran blue solution was pipetted on to the top of the column and the effluent volume collected until the dye was eluted from the outlet. This was 58 mls. = V_o.

The U.V. absorption is seen in Fig. 8.0 λ max. are seen at 215 and between 260 - 275 mμ.

Assuming proteins eluted in the 1st peaks below are completely excluded from the gel their elution volume represents the void volume.

Application of plasma sample

Plasma may be applied to the top of the column in two ways both of which were used.

1) Sucrose 10-20% may be added to the plasma sample to make it more dense than the buffer. It is then added either by pipette, or more easily and reliably by pumping on to the surface of the gel (separated by a circle of filter paper) but below the surface of the buffer.

2) The superfluous buffer above the level of the gel surface may be removed and the plasma sample gently placed on top of the filter paper. The outlet is kept fully open through this procedure. When the plasma has entered the top of the column, buffer is gently added (2-5 mls.) and this level maintained throughout the separation.

Buffer choice

Because of the risk of denaturation and precipitation of the proteins in the column, an electrolyte buffer was used as eluant to prevent this. The buffer used was 0.1 Tris and 1 M sodium chloride. This molarity of sodium chloride was used in preference to a more dilute solution in order to minimise protein protein interaction.

0.1 M TRIS HCl pH 8.0 = 12.114 gms/L. Tris. The pH was made 8.0 by adding conc. hydrochloric acid. 58.4 gms/L of sodium chloride was added to give a 1 M solution.

Plasma samples As mentioned previously the object of this separation technique was to obtain as rapid a separation with as great a fractionation as possible. If too much plasma is used initially the high flow resistance due to viscosity leads to diffuse zones with obvious poor fractionation.

5 mls. plasma was used in the first separation but later 3, 2 and 1 ml. samples only were used. The use of the latter volume had an additional advantage in that dilution of

the eluant was not needed to obtain U.V. absorption as an accurate scale at 280 mu.

Column and separation details

The column was water cooled to 3-5°C and the top of column was 6'8" above the fraction collector to give adequate hydrostatic pressure and consequently reasonable eluant flow rates.

Results G 200 column : V = 90 mls. (3 gms. G. 200) Fig. 8.1.

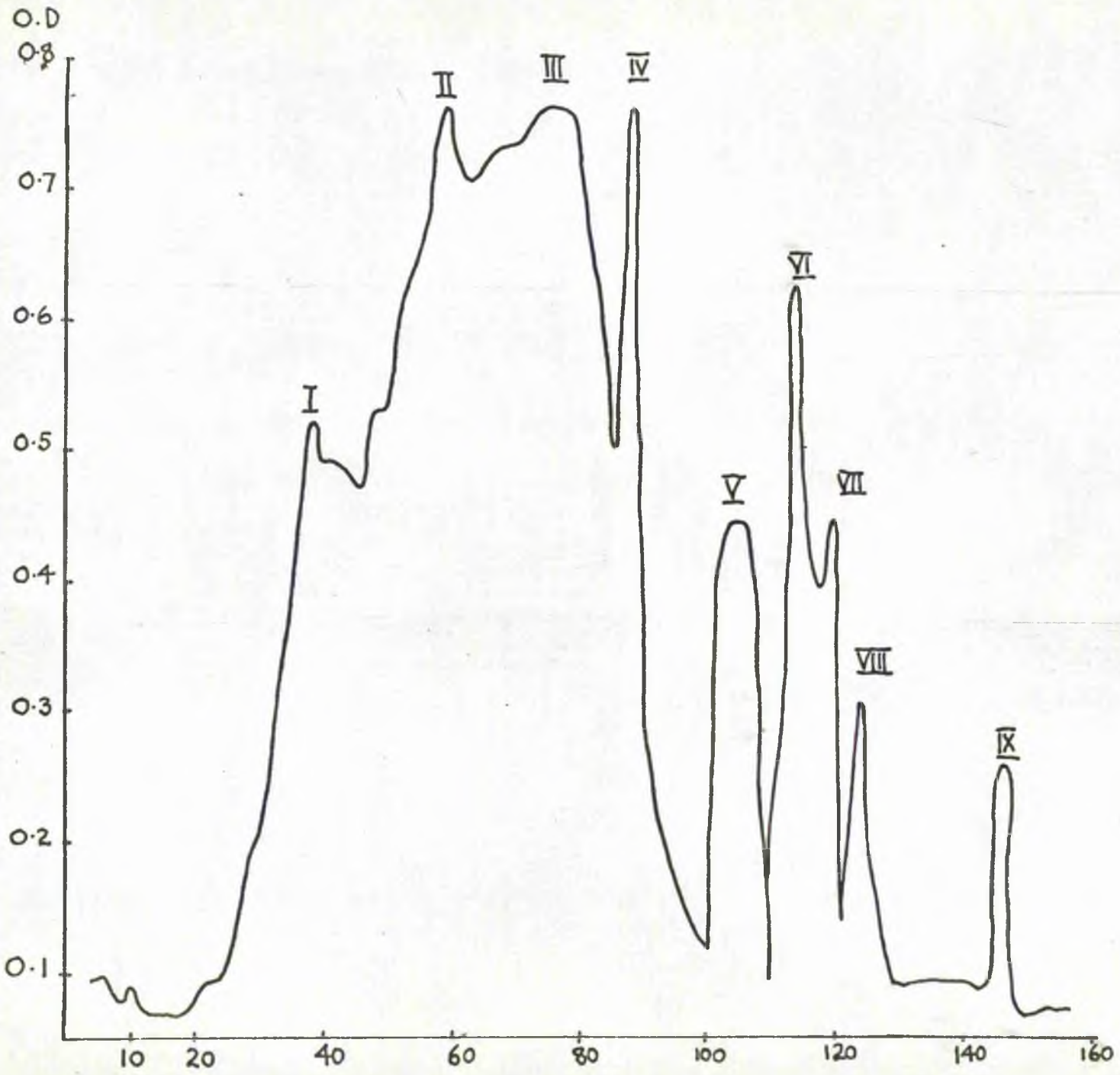
I To 5 mls. plasma was added 0.5 gm. sucrose which was dissolved by gentle rotation. This was layered on the column by a micro pump over 12 mins. beneath the surface of the buffer. 30 minute fractions were collected of 2-4 mls. The volumes were kept as near as possible to 3 mls. Owing to the protein concentration in the eluant a dilution of 10 times with buffer was necessary before the optical density at 280 mμ could be read.

The results are seen in Fig. 8.1.

The total volume required for complete elution was 134 mls. The volume needed to initiate protein elution from the column was 30.25 mls.

Peaks, I, II and III required 50, 31 and 22.05 mls. respectively for complete elution.

FIG. 8·2 Fractionation of Plasma control. G.200



This separation was repeated with a control sample from an operation with very similar results. The fractionation however was slightly worse and therefore a larger column was set up to try and improve this factor.

II Fig. 8.2: <u>Fractionation of 1 ml. plasma</u>

A 2 x 80 cm. column was filled with equilibrated G 200 (6.7 gms) so that V = 220 mls.

To 1 ml. plasma (control) was added 0.1 ml. sucrose and the separation repeated as above. The elution rate was 2-4 mls/30 mins. A vast increase is seen of at least 9 peaks. The significance of these will be described later.

The volumes of each fraction were thus :

"Void volume" up to first protein elution = 36 mls.

Fraction I = 50 mls.

II = 38.5

III = 35.5

IV = 22.0

V = 11.0

VI = 26.0

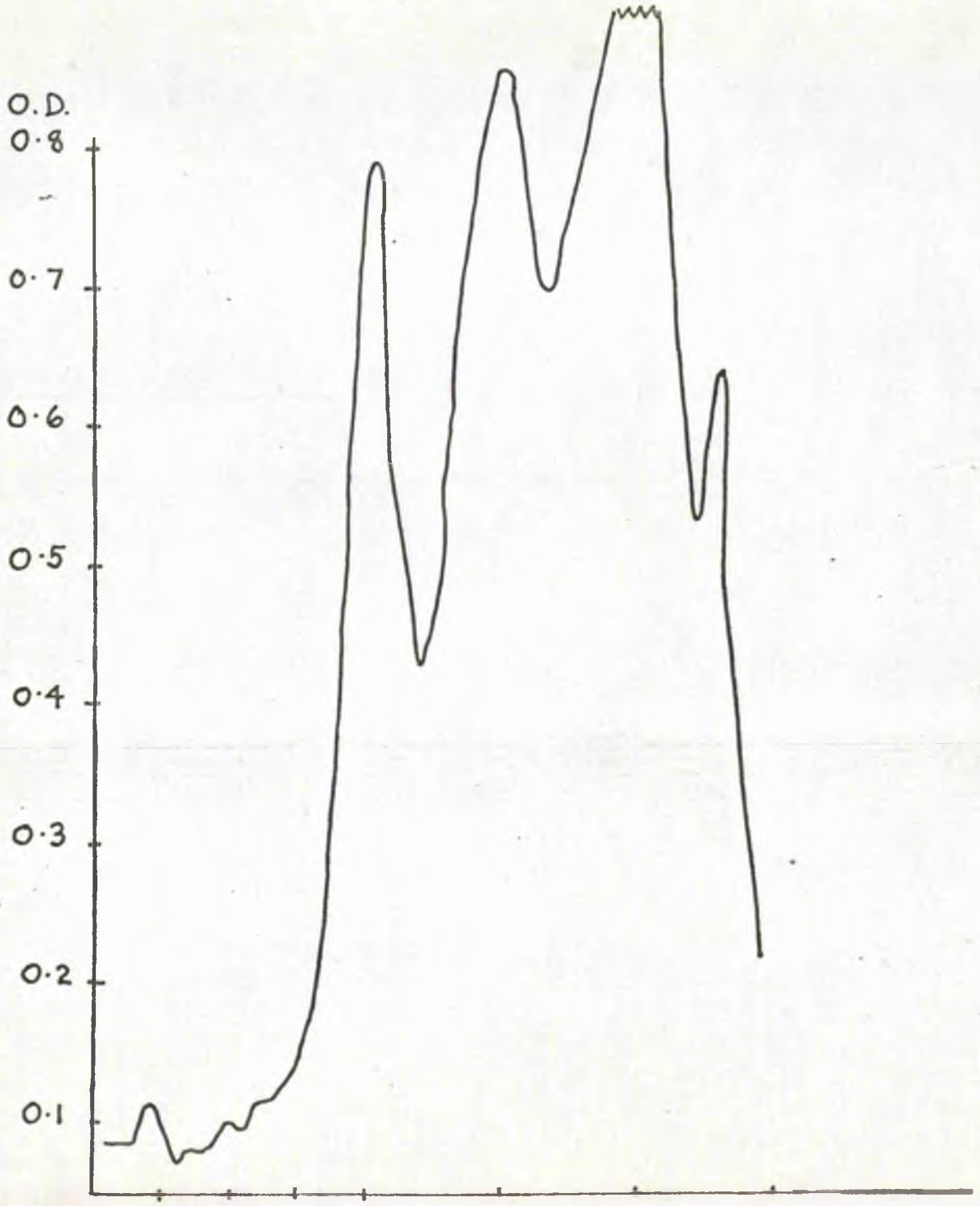
VII = 5.0

VIII = 32.0

fraction IX = 15 mls.

FIG. 8·3

Fractionation of Haemolysed Plasma (operation)



Plasma - haemolysed from operation. Fig. 8.3.

A plasma (haemolysed) specimen from the end of a full perfusion operation was separated on this column in the same manner as above. 0.1 gm. sucrose was added to 1 ml. plasma to improve density of sample. The results were very similar in the beginning, to those above, giving the four first peaks. Unfortunately the fraction collector broke down at this point and due to loss of protein through overflow of eluant this experiment was terminated.

Approximately 32 mls. of eluant was required before the major protein peak (I) began to be eluted. It is interesting that a very minor peak occurred in this first eluant volume and is consistent with findings in the other fractionations. Great care was taken to ensure this was no artifact, i.e. due to incomplete protein elution from a previous separation.

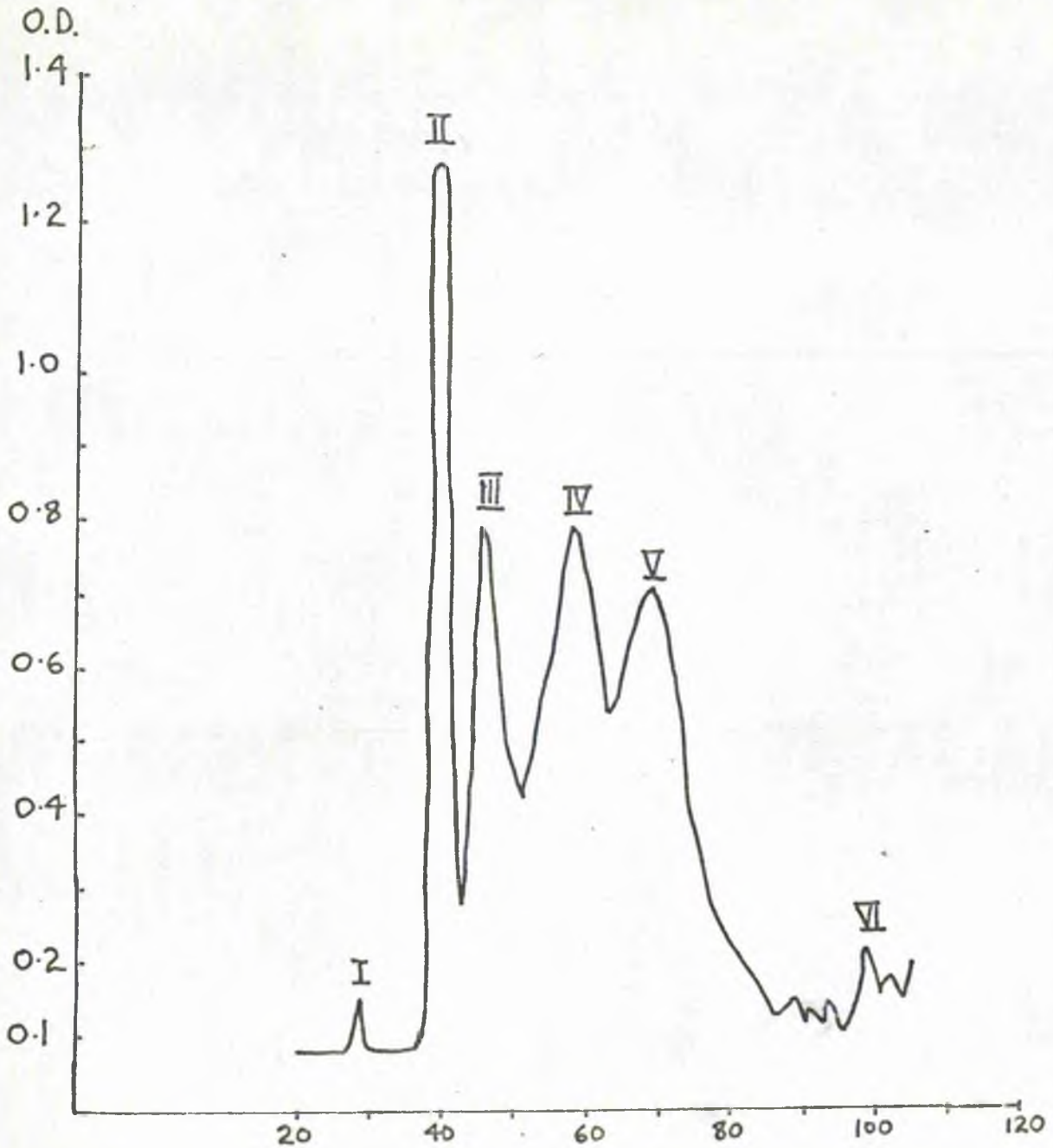
The fraction volumes were thus.

Fraction I 48 mls.
" II 34.6
" III 31.6
" IV 16.0

other fractions were unobtainable.

As the fractionation of haemolysed plasma from operation was unable to be repeated, the following experiment was performed to contrast both with this operation sample and the control plasma sample.

FIG. 8·4 Fractionation of Denatured Haemolysed Plasma.



Fractionation of haemolysed denatured plasma. Fig. 8.4.

Fresh blood was shaken vigorously for 4½ hours in the presence of 6.64 M urea, (8.0 gm. urea was added to 20 mls. of blood). This was centrifuged and the plasma separated.

The degree of haemolysis was estimated by the cyancmethaemoglobin method (0.05 mls. plasma + 0.05 mls. saline 0.9%). The absorption at 540 mu was 0.049 this is equivalent to 7% haemolysis approximately. The U.V. absorption of this 1% plasma in haemoglobin reagent gave similar results to those seen in Fig. 8.5. The results of this U.V. absorption of plasma in the haemoglobin reagent are as follows.

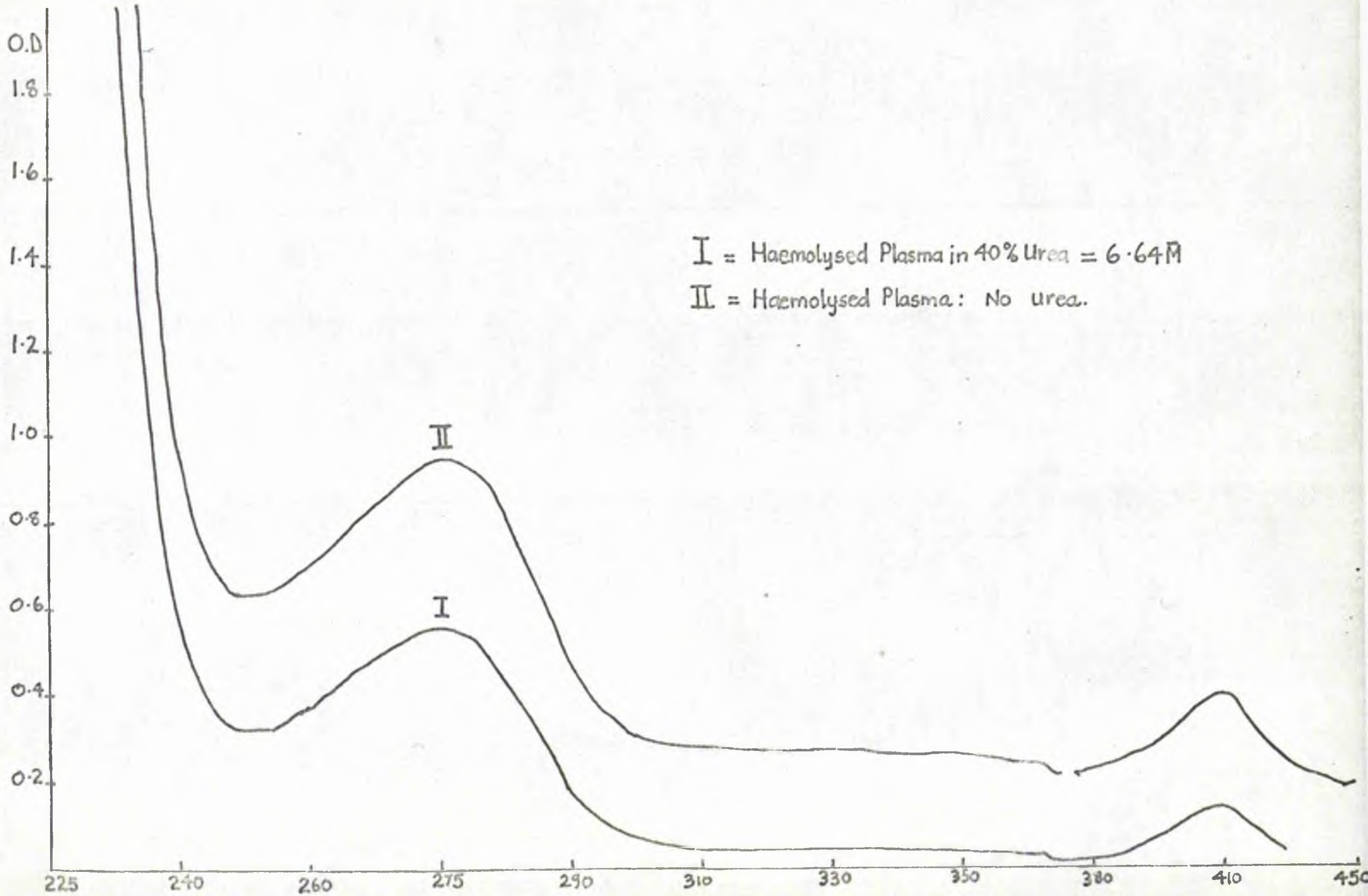
1) Protein peak at 280 mμ = 0.725 O.D. (1% plasma = 0.82 mgms/100 mls.

2) Peak at 410 mμ due to haemoglobin = 0.235 O.D.

Thus the cyancmethaemoglobin result of 0.049 at 540 mμ = 0.235 at 411 mμ. Non haemolysed plasma does not absorb at 410 mμ. If one assumes an approximate 15 gms. haemoglobin/100 mls. blood this degree of haemolysis is equivalent to just over 1000 mgms of free haemoglobin/100 mls. blood. This is greatly in excess of that found in cardiac operations.

The plasma protein concentration was 8.2 gm/100 mls. measured at 280 and 260 mμ.

FIG. 8·5 U.V. absorption of Haemolysed Plasma.



The 1% haemolysed plasma with and without 40% urea (6.64 M) was scanned in the U.V. spectrum (Fig. 8.5); other than a minimal increase in the 280 and 410 mμ peaks there is no visible change.

1 ml. of this denatured haemolysed plasma was fractionated on the G.200 column. A markedly different separation was obtained consisting of 5 major peaks and a sixth fraction containing multiple small peaks. Again in the plasma protein "void volume" the very minor protein peak is again observed.

The fraction volumes were thus :

Fraction	I	30.0 mls.
	II	21.4 "
	III	26.2 mls.
	IV	40.0 mls.
	V	43.3 mls.
Fraction	VI	25.3 mls. +

Discussion

In this introduction mention was made of the care which must be taken to ensure that excessive packing of the column does not take place. Presumably due to the length and volume of the second column, it was found that elution times gradually lengthened. This last fractionation took 5-6 days. It would appear from these facts that it

may well be wise in future to use smaller columns and to refractionate crude separations either through the same column or use a different column e.g. the DEAE Anion exchanger.

The use of both columns where V_t = 90 and 220 mls. did show however greater separation of the plasma proteins than was at first expected. From the results of electrophoresis and ultra centrifugation it would be expected that only 3 main peaks would be separated viz:

large α and β lipoproteins M.W. 1,300,000

1) Macroglobulins α_2 + β_2

+ other 19 S components e.g. Cold agglutinins.

2) γ globulins M.W. 160,000 approx. 7-9S

Anti-Rh. anti-D and anti-A globulins.

α + β globulins

? Transferrin.

3) Albumin M.W. 70,000 3-4S components.

α_2 globulins and α_1 glycoprotein

? Transferrin.

The order of protein elution therefore that would be expected is one of decreasing sedimentation coefficients with increasing degrees of retention on the column.

In the case of haemolysed plasma the free haemoglobin would be expected to be eluted either before, with, or after albumin - on a molecular size basis.

It is interesting that in the plasma protein separations performed in this section the elution volume was as low as 4-8 mls. per hour and that in spite of only using 1 ml. plasma, complete separation of proteins took 4-6 days. These facts are in marked contrast with those criteria of Killander and Flodin who using a 4 x 40 cm column (G 200) and 8-10 mls. serum completed elution of proteins in 5 Hrs!

As the separations seen in Figs. 8.1 - 8.4 show - more than 3 peaks were obtained by 1) slow eluant rate 2) using minimal plasma volume. It is interesting that the first fractions contained the greatest protein concentrations and the latter fractions, least.

As has been mentioned, emphasis in this preliminary protein separation has been to find the optimal criteria required for maximal plasma protein separation.

The fractions obtained in Figs. 8.1-3 inclusive bear many resemblances in spite of the fact that 1 and 2 are control plasmas and 3 is a haemolysed plasma. Apropos of this it is of interest that Flodin and Killander in one of their serum separations added haemoglobin to serum in excess of the haemoglobin - binding capacity of the haptoglobin contents of the mixture. Both sera belonged to the haptoglobin group 2-1. The haemoglobin in the fractions, detected by using the benzidine reaction, was found in two parts of the three main peaks.

1) At the rear part of the first protein peak, and

2) At the rear part of the last protein peak.

This signifies that part at least of any free haemoglobi in plasma is not eluted pari passu with albumin but is bound to part of the globulin moieties. This increases the moecular weight which reduces even further any tendency to be absorbed through the gel pores. Thus elution is more rapid than the lower moecular weight proteins. Since the haemoglobin present in the plasma in Figs. 8.3 and 8.4 were of the order of 300 and 1000 mgms/100 mls. respectively it is reasonable to suggest, in the absence of further investigation, that the larger peaks eluted first, may well contain some haemoglobin.

In Figs. 8.1 and 8.2 the first protein fractions are still large when the plasma contains virtually no free haemoglobin. This would suggest that the free haemoglobin present in Figs. 8.3 and 8.4 if present in the initial peaks is of small concentration.

The very minor peak seen just before the elution of the first major fraction is a constant feature in all separations. It is most likely an α or β lipoprotein or γ globulin. Its constancy in all separations would suggest - if indeed it is a lipoprotein - that cleavage due to denaturatio

is not occurring to any significant extent during cardiopulmonar perfusion, into its constituent lipid and globulin moieties.

Further identification of fractions from this initial separation may be achieved after pooling, concentrating and dialyzing by using such procedures as paper chromatography, sucrose density gradient ultracentrifugation, analytical ultracentrifugation, immuno electrophoresis, double diffusion in gel technique, antibody titrations and partial neutralisation studies.

The 3 primary peaks found by Flodin and Killander, 1962 consisted of the following fractions and sedimentation coefficients.

First Peak - Fraction I α_2 globulin fast γ globulin Anti A activity α-β lipoproteins $\alpha_2^+ \beta_2$ macroglobulins	19 S (10-11 S)
α protein (not identified	
19 S antibodies	
A 7S lipoprotein	

Second Peak - Fraction II 7 S and 4 S fractions

γ globulin

also ? albumin, α_2, β, and β_2 globulin mobility fractions

Anti A activity

7 S antibodies.

Third Peak - Fraction III

Albumin

α_1, α_2, and β_1 globulin } 4 S

The transferrin detected in fraction III was eluted just in front of the albumin. The α_1, globulin in this fraction is probably the α_1 glycoprotein (3.5 S).

Albumin and γ globulin present in high concentrations were found in small amounts in fractions some distance from their respective maxima. This suggests some protein - protein interaction may have taken place owing to use of buffer of only 0.2 M NaCl. This interaction is minimised by using buffers of higher ionic strength. For this reason in Figs. 8.1 - 8.4 a Tris buffer was used containing 1 M NaCl.

In the first peak of Flodin and Killander a fraction was obtained which sedimented at a 7 S value. This was most likely a lipoprotein of less density than other serum proteins,

and in spite of its large size, sediments at a slower rate.

CONCLUSION

The emphasis has been on the preliminary separation of plasma proteins rather than absolute quantitative changes of individual fractions.

1) Fractionation of plasma proteins by column chromatography with G 200 shows distinct changes between control (9 peaks) and denatured plasma (6 peaks).

2) Haemolysed plasma from perfusions were not available during these fractionation studies (except the one mentioned).

3) Faster separations would be beneficially obtained using a more extensively cross linked gel e.g. G. 100, together with a second column e.g. the D.E.A.E. Anion exchanger with ancillary methods such as :-

(a) paper chromatography

(b) Sucrose density gradient ultracentrifugation

(c) Analytical ultracentrifugation

(d) Immuno electrophoresis

(e) double diffusion in gel technique

(f) antibody titrations and

(g) partial neutralisation studies.

SECTION 9 HORMONAL CHANGES IN CARDIAC SURGERY

Introduction

As mentioned in the initial introduction (Section I), it became increasingly important as well as interesting to find out if the small increases in plasma protein denaturation, haemolysis, viscosity, turbidity, and amino acid changes as well as the host of other criteria not being examined critically in this work (e.g. pH changes, electrolytes, physical trauma), - were giving rise to significant stress as measured by hormone methods.

Emphasis has already been made in Section I of the importance of analysing as many criteria as possible (in patients on full perfusion) in order that more exacting conclusions may be drawn from the experiments performed and the results obtained.

Stress (in this case caused by prolonged operations and oxygenation of the blood) is the physiological adaptation of the body to an adverse environment. Shock occurs when compensatory mechanisms (nervous, cardiovascular and hormonal) can no longer cope with this adverse environment.

It was the initial idea in this hormonal work then, to compare the adrenal response directly with the other criteria mentioned above and in other sections, especially amino acids.

Initially it appeared that by using an A.C.T.H. (corticotropin) assay procedure the problem of measuring stress in absolute terms would be solved. It became rapidly obvious that due to the following practical difficulties it is far wiser, easier, less time consuming, and just as accurate and valuable to measure the 17-hydroxycorticosteroids of the blood in lieu of ACTH.

ACTH Assays

The determination of ACTH in plasma demands an extremely high degree of sensitivity and good precision on the part of the bio assay method.

In the past the adrenal ascorbic acid depletion technique was only available and was not sensitive enough to detect ACTH in a concentrate equivalent in volume to 40 mls. blood (normal). More recent methods of detecting plasma ACTH in normal subjects are only minimally better.

Purves and Sirret, 1965 have assayed ACTH in dexamethasone treated rats - this drug in fact carries out a chemical hypophysectomy overnight.* Next day a small human plasma sample is injected into the external jugular vein of the rat. 18 minutes later a blood specimen is withdrawn from the same vein and estimated for corticosterone.

*This is dissolved in the drinking water.

I.W.S. standards are used in the initial stages. The difficulties with this procedure - besides strict control of laboratory animals - are the necessity for absolute cleanliness in reagent preparation and technique, which is time consuming and the necessity of a Flucrimeter for the actual assay. Also a major difficulty is that ACTH must be assayed within 30 minutes of being withdrawn from the patient (pump circulation). Logistics would not allow this.

Added difficulties with ACTH storage are :

1) Blood or plasma stored in glass bottles rapidly looses it's ACTH activity (30 mins. - 2 hrs.) through some unexplained mechanism. Mercoptoethanol appears to minimise this. Plastic tubes are no better and to date the substitution of siliconised, soft flint glass for hard borosilicate glass is recommended, (Yalow and Berson, 1969).

2) In normal human plasma the concentration of ACTH is very low (0.25 - 0.7 mu/100 mls. plasma) and only in pathological conditions of the adrenal and pituitary are high levels observed, e.g. adrenocortical insufficiency, Addisons disease, adrenalectomy, and Cushings syndrome.

Other bio-assay methods similar to Purves and Sirrett above are

1) The adrenal vein corticosterone estimation in hypophysectamized rats (Lipscomb and Nelson 1962). Standard

ACTH injections are equated with corticosterone concentrations in the adrenal vein, and measured as above by the flucrometric method. This would indeed be the method of choice if the samples of blood from open heart surgery could have been analysed within 20 minutes. This unfortunately was out of the question due to the work of this volume being carried out at a different locus.

2) Sayers, Sayers and Woodbury, 1948 have used the rat 24 hours after hypophysectomy in an adrenal ascorbic acid depletion technique. The left adrenal after hypophysectomy is removed, weighed and assayed for ascorbic acid. The material to be assayed is then injected into the rat and one hour later the right adrenal removed, weighed and the ascorbic acid concentration determined. The difference in the ascorbic acid levels is directly proportional to the dose of ACTH injected.

This method is less sensitive than Lipscomb and Nelson, 1962.

3) Nelson and Hume, 1955, have measured the 17-hydroxy-corticosteroids from the adrenal vein in a hypophysectomised rat, before and after injection of standard or unknown ACTH sample. Although the index of precision is good, it has, like the above method low sensitivity.

4) A similar method to Nelson and Hume is that of the plasma corticosterone assay of rats (hypophysectomised) peripheral blood before and after ACTH injection, by Guillemin et al., 1958. Fluorescent steroid is measured rather than corticosterone.

5) A similar method to Sayers et al. is that of Munson and Teopel, 1958. Here the increase of ascorbic acid in the adrenal vein is measured before and after ACTH.

6) Vernikos-Danellis et al. in a recent method 1966 has assayed the fluorescent steroid in a hypophysectomised rat. The adrenals are removed in the same manner as Sayers et al. method, before and after ACTH loading.

In a personal communication, Davies at Mill Hill developed a method virtually the same as Vernikos-Danellis, Silber et al., 1958, and Lenker and Bernstein (1958).

Of all these methods, variations of the same theme removal of adrenals compared with cannulation procedures is simple, less tedious and gives the same degree of resolution and sensitivity.

The last method to be mentioned of completely different technique is that of immuno assay. The theory is based on that of the well known insulin I.A. method. ACTH is assayed by its ability to compete with I^{131} labelled ACTH for binding sites on an antibody made against ACTH in a guinea pig.

As the concentration of unknown (ACTH) increases the ratio between the free ACTH and the antibody bound ACTH diminishes.

This is the method par excellence, developed by Yalow et al 1964 Yalow and Berson, 1963 have recently discussed the difficulties and pitfalls of ACTH assay which include.

1) The main problem - the fact that ACTH occurs in low concentrations in human plasma.

2) The marked susceptibility of ^{131}I-ACTH and ^{125}I-ACTH to damage in the plasma during the incubation period of assay.

3) The annoying feature of adsorption of the hormone to glass, with loss of activity has already been commented upon.

4) The "decay catastrophe" of labelled molecules is a condition in which the latter eventually and inevitably undergo physical decay of the radioisotope, this is due to the requirement for high specific activity of labelled hormones when the molecules are labelled with more than one radioactive atom.

5) There is an enzyme system in plasma which degrades ACTH, and which only seems to be active after storage.

It is of interest that in the bio assay of human plasma ACTH using rats, as much as 4 mls. of this plasma (pH 7.2) may be injected without "apparent" repercussions.

Normal human plasma contains ACTH in an approximate concentration range of 5-50 μμg/ml. (Berson and Yalow). The normal range has not yet been determined accurately but it very likely is within 0.25 - 0.7 mu/100 mls. plasma. The former value being that of Ney et al., 1963.

Relation between ACTH and corticosteroid secretion

In 1943 the circadian rhythm of adrenal corticosteroid secretion was noted by Pincus; and Fagin (1969) drew attention to the circadian periodicity of blood amino acids in normal and adrenalectomized mice. Plasma levels of 17-hydroxycorticosteroids (17-OHCS) are highest in the early morning 5-6 am and fall sharply before noon, remain low throughout the afternoon and evening and are at the lowest level about midnight, after which the concentration begins to rise.

The ACTH concentration at 6 am is approximately twice that at 6 pm (Ney, 1963).

ACTH secretion follows a very similar pattern but it must be emphasised that a very small change in the plasma ACTH secretion (measured in μμg/ml or m.u./100 mls.) has a marked effect on the adrenal corticosteroid secretion which is measured in μgm or mgm units. From the point of view of measuring corticosteroid secretion during open cardiac surgery it should be borne in mind that normally the secretion

is gradually decreasing over the 10 a.m. - 11 p.m. period so that any increase which is found is in fact of all the more importance.

The mean ACTH concentration at 8-10 a.m. is 22 μμg/ml. and before midnight this value is down to 10 μμg/ml.

1 μμg/ml. ACTH = 0.00141 mU/ml.

The question of how much the plasma ACTH concentration must be raised in order to increase the adrenal corticosteroid secretion was concisely answered by Liddle et al. in 1962.

An infusion of ACTH of 0.05 u/hr. gave the following mean results.

Plasma ACTH 0.2 mu/100 mls.

Plasma 17-OHCS 22-27 μg/100 mls.

Urinary 17-OHCS 15 mgms/24 hrs. (normal 3-12 mg.)

These facts are of great importance when investigating ACTH, and/or corticosteroid secretion in abnormal conditions e.g. trauma, surgery, Cushings Syndrome and many others.

1.0 mu ACTH/100 mls. plasma is only just detectable by the most sensitive assay procedure when whole plasma is injected into test animals. Concentrations of 0.2 - 1.0 mu ACTH/100 mls. plasma cover the range of adrenal cortical hormones from normal or slightly above normal to far in excess of normal.

It is most unlikely therefore that when assaying ACTH concentrations as a guide to the degree of stress - in this case prolonged surgical trauma - any change - if at all - will be found. Cooper and Nelson, 1962 investigating ACTH changes in surgical patients found that in only 3 out of 10 were control values detectable, (0.6, 0.6 and 0.9 mu ACTH/100 mls. plasma).

The above data can be expressed in another way to reinforce this fact.

A small and most likely <u>undetectable</u> increase in ACTH secretion induces a significant and <u>measurable</u> increase in adrenal cortical hormone release.

In the operations studied therefore the plasma 17-hydroxycorticosteroids were estimated with interesting results. The opportunity arose to study plasma samples in operations in which hydrocortisone (100 mgms/hourly) was given routinely, and also in operations in which it was purposely withheld thus giving an interesting comparison.

It is worth emphasizing that 17-hydroxycorticosterone, cortisol and hydrocortisone are the same steroid.

Sayers, 1948 has pointed out that although surgical trauma increases plasma ACTH concentrations at least three fold, the elevated values are still below the sensitivity of

all but the most sensitivie methods of bio assay (probably radio immuno assay).

It is of interest and importance as to why hydrocortisone is used in cardiac operations. This will be elucidated in the discussion. 17-hydroxycorticosterone was estimated in the plasma since it is the chief circulating C_{21} adreno cortical steroid of man, which is in appreciable concentration in the blood, 6-25 µgms/100 mls. plasma.

Casey and Zimmerman, 1957, in their investigation of aldosterone excretion in the post operative patient found that although both urinary aldosterone and 17 OHCS are increased post operatively, the former showed a transient excretion persisting no longer than 2 days, and showing no affinity with the period of positive sodium balance which lasted 7 days or so. Aldosterone therefore appears to have only an initiating effect on sodium conservation. It has no association with the pituitary and only small quantities of aldosterone are released following administration of large doses of ACTH.

It has been postulated that the right atrium may be concerned in sodium balance, as well as the kidney, Shu'ayb et al. 1965, but aldosterone would still have its major effect on the latter.

METHOD

17 hydroxycortic steroid estimation in plasma

The following method was based on that of Peterson, Karrier and Guerra, 1957, but with modifications.

The steroid was extracted from plasma - (which was virtually in the fasted state) with an organic solvent and the extract washed with an aqueous base. A violet colour was developed in the organic layer and a correction made for interfering substances.

It is of the utmost importance that all reagents should be very pure, just as for the reagents needed in an ACTH bioassay. This is because impurities very greatly affect the final readings and therefore the initial calibration curve.

For this reason the preparation of the reagents will be described here instead of in Appendix I.

Reagents

1) Dichloromethane (methylenedichloride)
2) 65% sulphuric acid
3) 0.1 N sodium hydroxide
4) Phenylhydrozine hydrochloride
5) Colour Reagent (phenylhydrozine-sulphuric acid-ethyl alcohol.

6) Hydrocortisonestandard

7) Blank Reagent.

Preparation of Reagents 1) dichloromethane

2-2½ litres of dichloromethane was divided into two volumes and each placed in a winchester bottle. To each was added 200-400 mls. of Analar concentrated Sulphuric acid. The bottles were shaken for a day on a Vigorous mechanical shaker. The volumes were then placed in a large separating funnel and the sulphuric acid fraction (lower) run off and discarded. This sulphuric acid has a yellowish colour. More Fresh Analar sulphuric acid was added and the whole process repeated for 6-8 days until the acid was colourless.

The dichloromethane was then washed twice with distilled water and twice with 1 N sodium hydroxide, and again twice with distilled water. The solvent was then dried overnight over anhydrous sodium sulphate and distilled slowly into a clean flask at 39-40°C.

2) Conc. sulphuric acid This reagent (Analar) was heated vigorously for 2-3 hours in a sandbath and cooled before adding 640 mls. to 360 mls. of distilled water to make 64 .

3) Sodium hydroxide A 0.1N solution was made up from the B.D.H. standard.

4) Phenyl hydrozine 100 gm. of this reagent was added to about 200 mls. water at 60-70°C.1 gm. of charcoal and 1 drop of conc. hydrochloric acid was added. This mixture was stirred for 2-3 hours at the above temperature. If 70°C is exceeded there is danger of decomposition.

1 Litre of boiling ethanol was added to the mixture and filtered while hot through filter paper. The filtrate was refrigerated overnight and the supernatant discarded next morning. The crystals formed were added to only 100 mls. of distilled water this time and the whole process repeated. This was finally repeated a third time with only 50 mls. of water.

The final collection of crystals was washed with cold ethanol (99-100%) and dried thoroughly. The crystals (fine, flaky and yellow) were stored in a brown stoppered bottle over calcium chloride.

5) Colour Reagent was made up daily. 50 mgms of pure phenylhydrozine hydrochloride was dissolved in 50 mls. of blank reagent.

6) Hydrocortisone standard 100 mgms of hydrocortisone was dissolved in 100 mls. absolute ethanol and kept in a tightly fitting screw top bottle at 2-4°C. 1 ml. was diluted to 200 mls. with water. This solution contains 5 µg/ml.

7) The Blank Reagent. 64% sulphuric acid was added to distilled water in the ratio of 2:1.

17-hydroxycorticosteroid assay (17-OHCS)

To 5 mls. of plasma (heparinised) in a 30-40 mls. glass stoppered test tube was added 25 mls. of dichloromethane. This was shaken vigorously for 15 seconds and allowed to separate. The plasma settles as the upper layer. As much of this layer was removed and discarded as possible with a fine ended pipette.

2 mls. 0.1 N sodium hydroxide was then added to the solvent layer and the tube vigorously shaken for 20 seconds. After allowing separation, the aqueous phase was discarded.

Two 10 ml. aliquots of the dichloromethane extract were transferred to two very clean glass stoppered test tubes - the latter is very important. They are best cleaned by water, ethanol and dichloromethane in succession. These two tubes serve as the "unknown" and "blank".

To one tube (unknown) 0.5 mls. of the colour reagent was added. To the blank - 0.5 mls. of the blank reagent was added. Both tubes were stoppered securely and shaken vigorously for 20 seconds. They were then left to stand for 30 minutes. The supernatant organic phase of each tube was then removed and the coloured (violet) aqueous phase left for 20 hours (8-24 hours) for maximum colour development.

Using a microsyringe the above volumes 0.5 mls. were carefully placed in microcuvettes the absorption read at 410 mμ. The spectrophotometer was zeroed with distilled water.

This entire procedure was repeated for

1) The Reagent Blank - 5 mls. of distilled water being used instead of 5 mls. plasma and

2) The Standards. The working standard contained 5 μg/ml. Serial dilutions of this were used for the calibration.

Since the microcuvette cells were not the absorption of each of them containing water was read at the commencement of the assay. They were numbered on the base to avoid any confusion.

The corticosteroid concentration was derived thus

1) From each actual reading the absorption of the cell itself was subtracted.

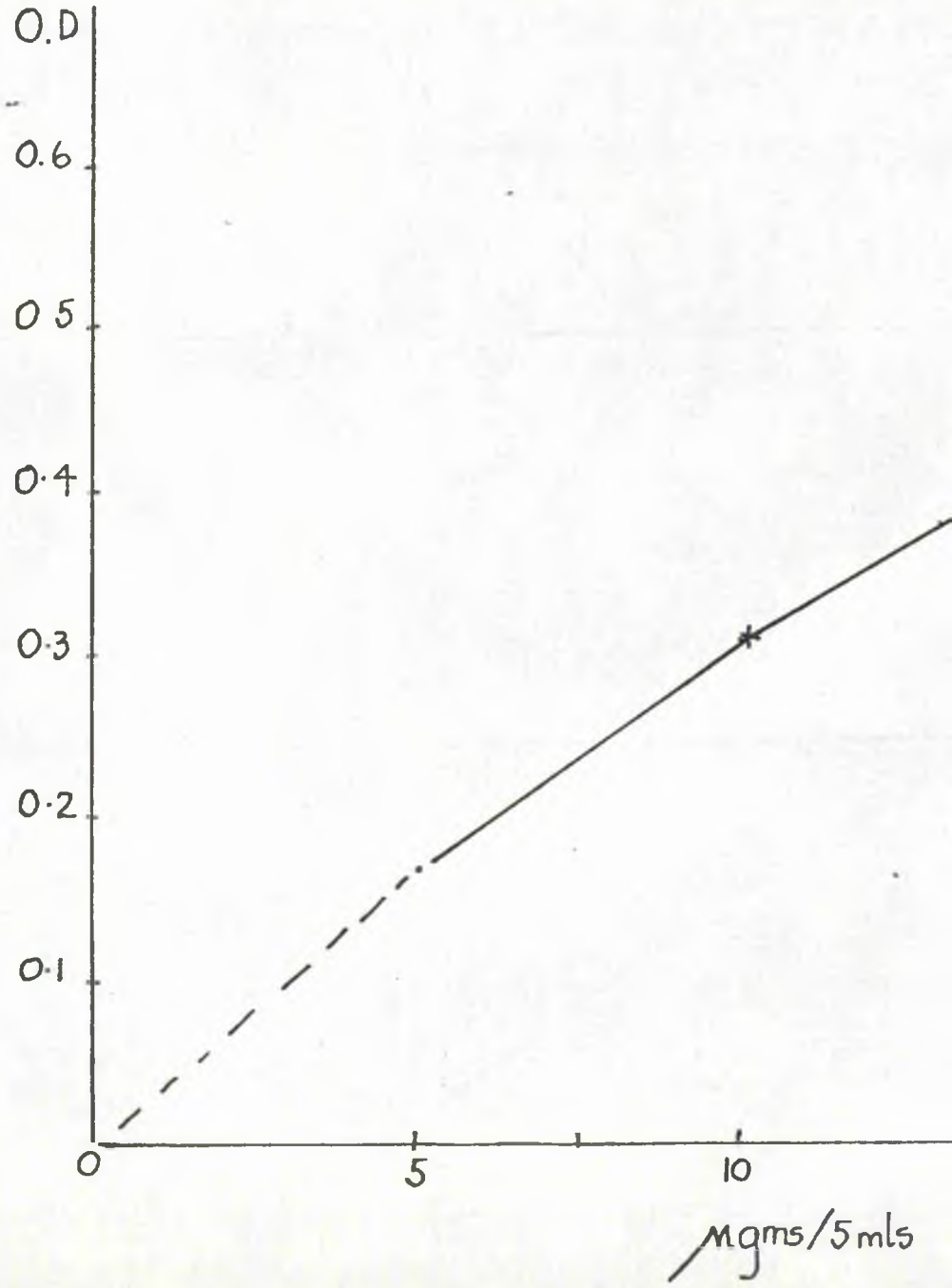
2) The C.D.P.S. = (Plasma sample - Plasma blank) - (Reagent blank - blank of the reagent blank).

3) The C.D.S. = (Standard-Standard Blank) - (Reagent blank - blank of reagent blank).

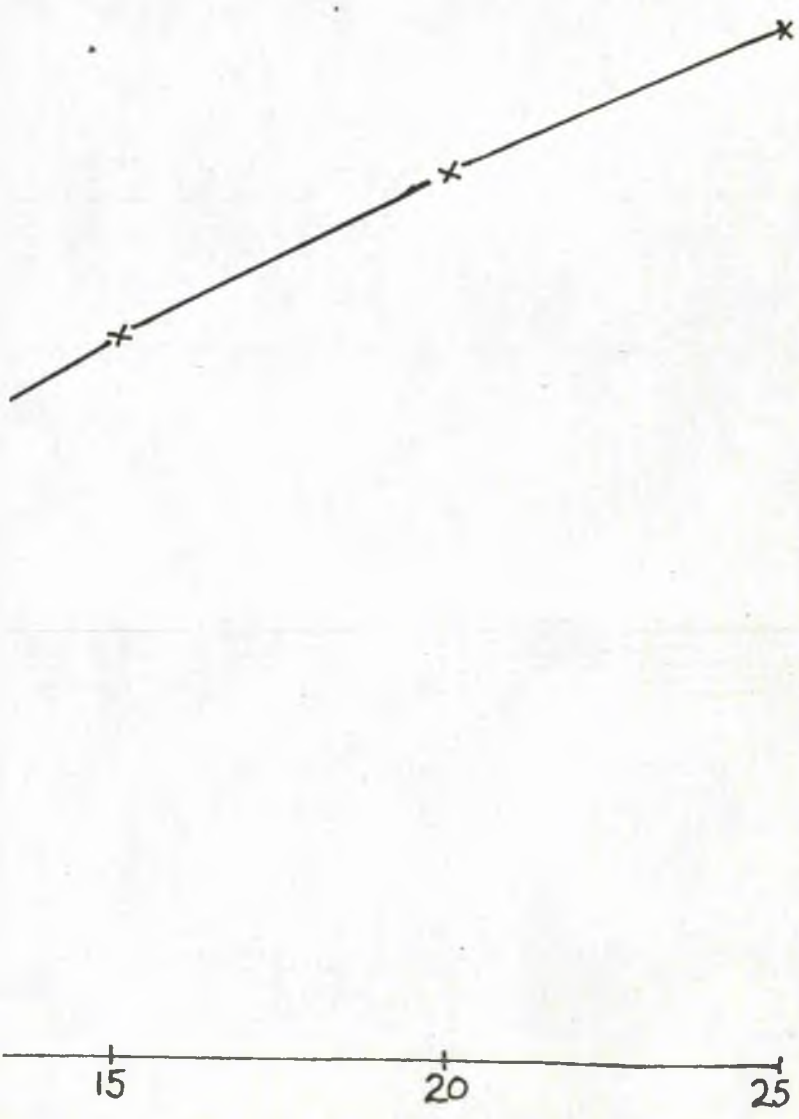
Thus : $\frac{\text{C.D.P.S.}}{\text{C.D.S.}} \times 100$ - μgm 17-hydroxycorticosteroid/ 100 mls. Plasma.

FIG. 9·1

17- Hydroxycorticosteroid



calibration @ 410 mμ.



where C.D.P.S. = Corrected density of plasma sample and

C.D.S. = Corrected density of standard.

RESULTS

A calibration graph Fig. 9.1 shows the results of standards 5-25 µgms.

After correction the results are as follows :-

	O.D. @ 410 mµ.
5 µgms/5 mls.	0.167
10 " "	0.306
15 " "	0.419
20 " "	0.521
25 " "	0.601

By this method normal plasma has a 17-hydroxycorticosterone content of 6-25 µgms/100 mls.

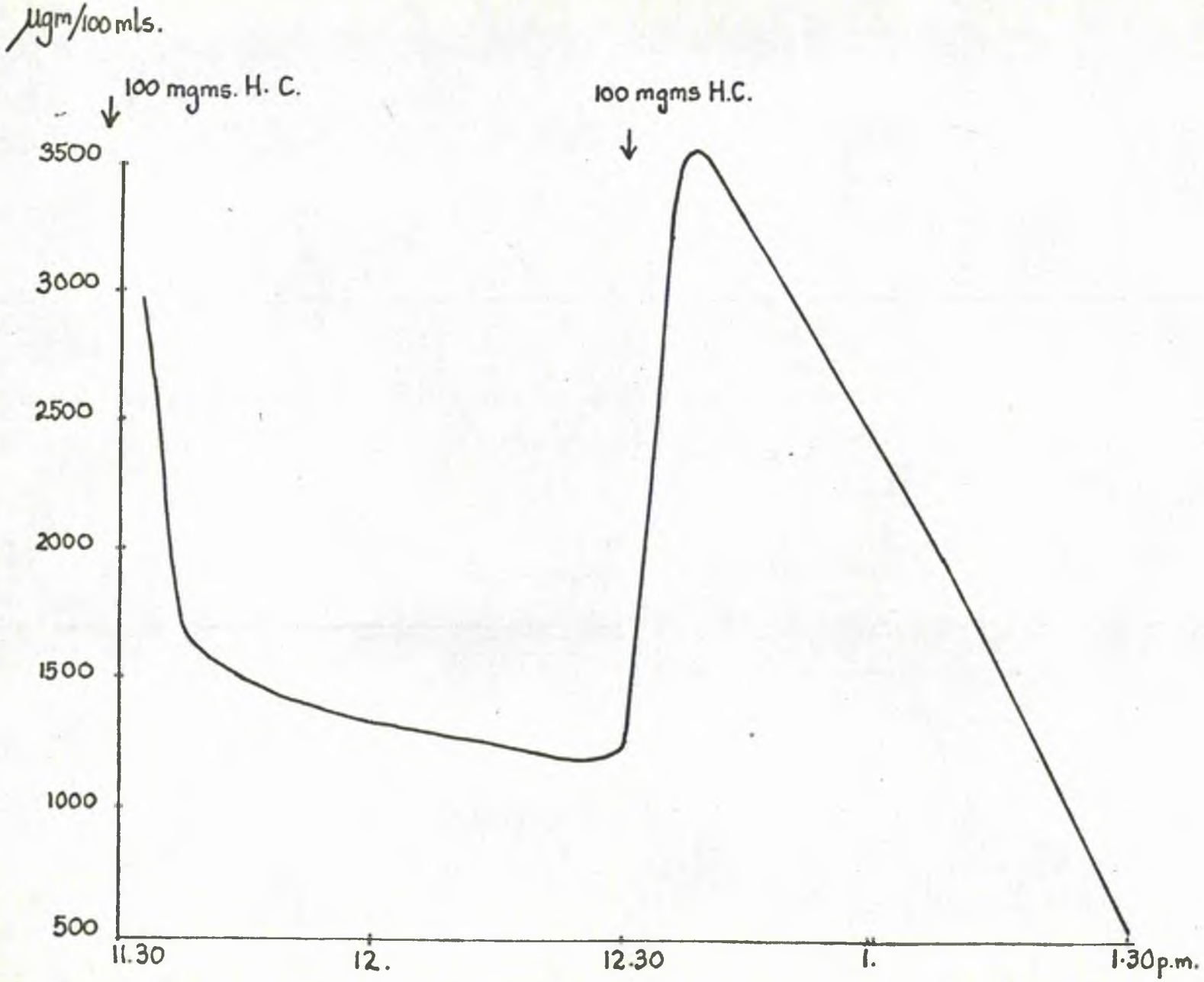
CASE I. R.M. Fig. 9.2 3 hrs. Full Perfusion.

In this operation 100 mgms hydrocortisone was given ½ and 1½ hours after commencement of full perfusion.

The following 17-hydroxycorticosteroid plasma concentration were obtained :

11.37 a.m.	1735 µgms/100 mls.
12.08 p.m.	1290 "
12.37	3530 "
1.08	1990 "
1.37	545 "

FIG. 9·2 Case I R.M.



CASE 2. A.C. Fig. 9.3 F.P.T. 2¾ hours.

Mitral valve replacement.

In this operation no hydrocortisone was added throughout the 2¾ hour full perfusion. As usual 5-10 minutes was allowed to elapse after commencement of full perfusion before the first blood sample was taken:

The results were

11.45 a.m.	(15 min. F.P.)	= 36 µgms/100 mls.
12.15	45 mins.	= 22.0 "
12.45	75 "	= 15.5 "
1.15	105 "	= 20.0 "
2 p.m.	150 "	= 24.0 "

CASE 3. D.S. Fig. 9.4 F.P.T. 30 mins.

Atrial septal defect.

Again this short operation had no additional hydrocortisone administered.

The 2 results were

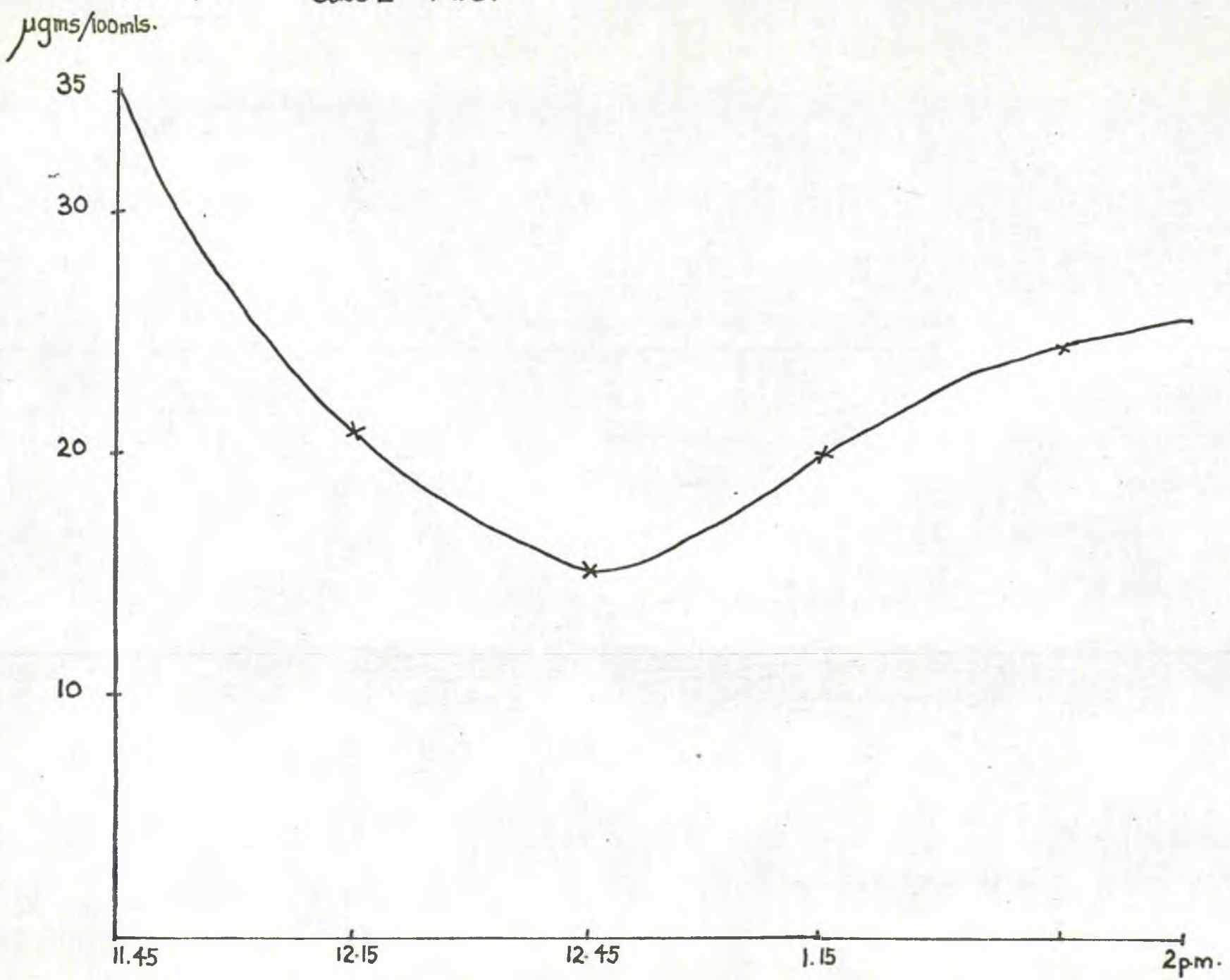
1) 18.0 µgms/100 mls.

2) 30 mins. - 25.5 µgms/100 mls.

CASE 4. R.D. Fig. 9.5 F.P.T. 2¾ hours.

Aortic valve replacement.

FIG. 9·3 Case 2 A.C.



No additional hydrocortisone

12.15	= 16.6 µgms/100 mls.
12.45	= 18.0 "
1.25	= 18.32 "
2.00	= 14.3
2.30 p.m.	14.25

For each assay reagent blanks and standards were taken through the whole extraction and reaction procedures pari passu with the plasma samples.

Discussion

These four cases are of practical interest for the following reasons.

1) The contrast between

(a) normal in vivo response of the adrenals to surgical stress and b) the effect of extrinsic (supplementary) hydrocortisone on this response.

2) The results show that surgical trauma does increase plasma 17-hydroxycorticosteroid concentration but only marginally, this may well be due to the fact that although the 17 OHCS are possibly substantially increased - the utilisation by the tissues is also increased. Therefore the net result is a lowered concentration (see p. 214).

FIG. 9·4 Case 3. D.S.

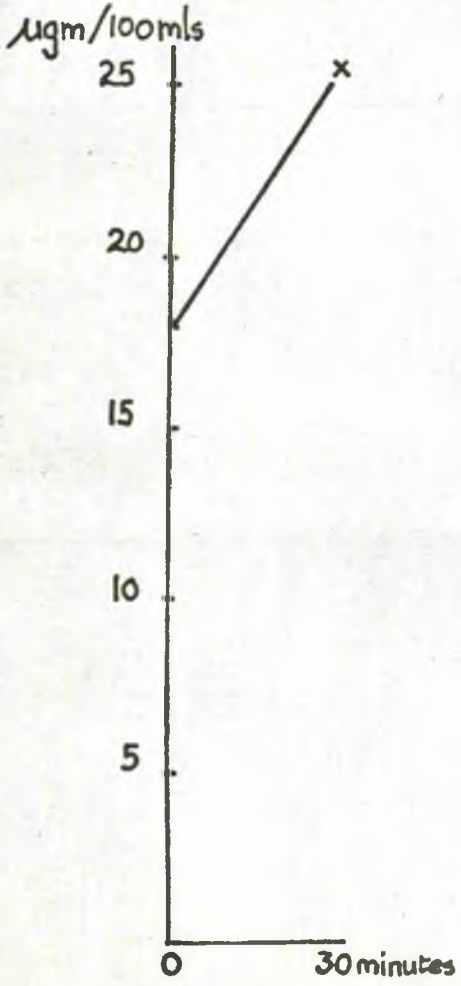
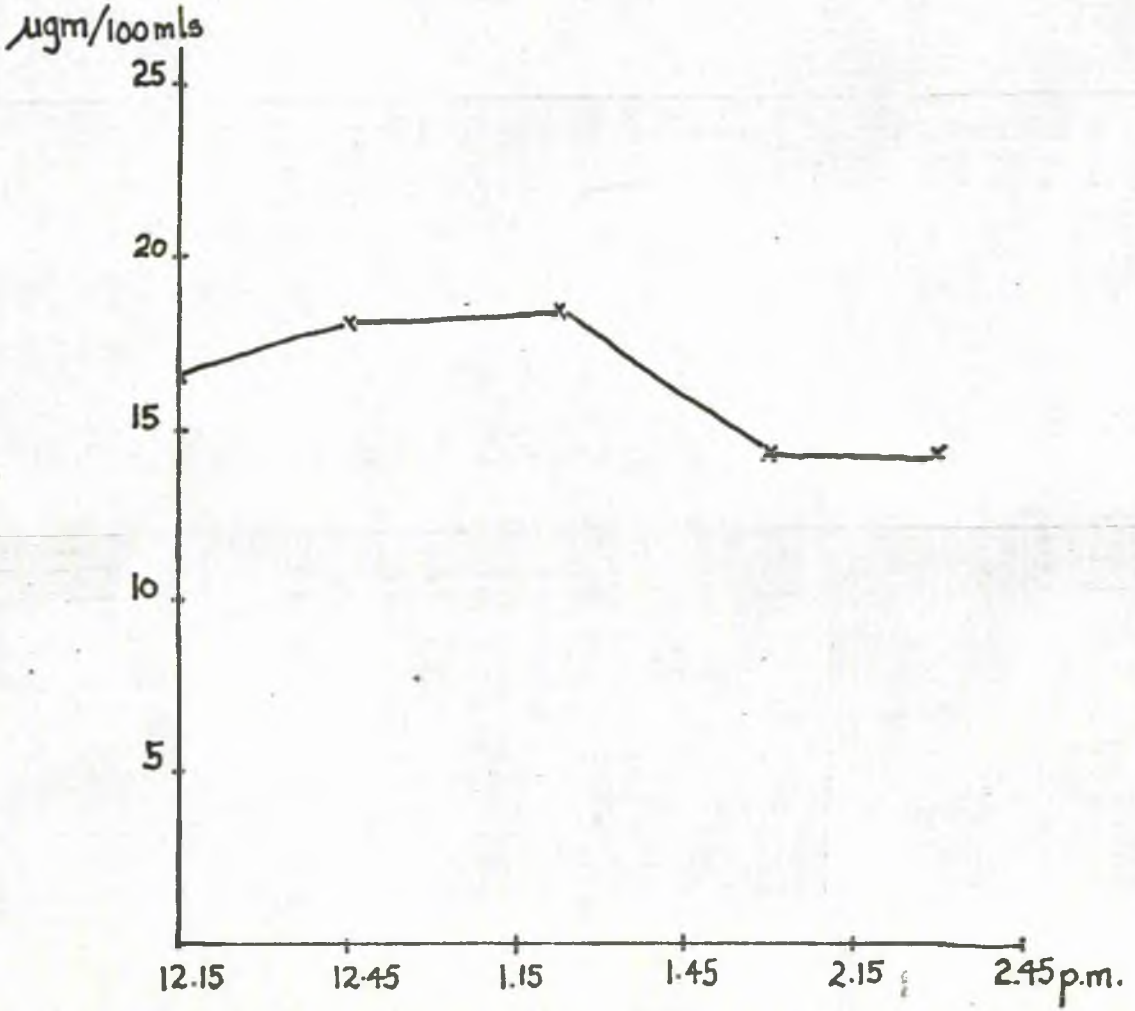


FIG. 9·5 Case 4 R.D.



In Case I the effect of 100 mgms hydrocortisone given routinely at hourly intervals is assessed. It was essential to find out whether this administration was purely empirical or whether indeed it had a vital part to play in "protecting" excessive adrenal response during prolonged cardiac surgery. In a personal communication with Ghadiali at the Brompton Hospital, London; hydrocortisone is given purely prophylactically for the following main reasons,

1) The Protective effect on coagulation factors
2) The Effect on capillary retraction and hence bleeding time.
3) The protective effect on the peripheral circulation and also
4) The protective effect on renal function during stress.

That surgical trauma does increase adrenal cortical secretion is well known (Casey et al. 1957, Smith et al. 1959, Cooper and Nelson 1962, Ney et al. 1963). The question in this work however was to find whether cardiac surgery gave rise to adrenal cortical secretion in excess of that normally found in general surgery.

Einerth et al. 1965, measured plasma corticosteroid concentrations in patients after accidental trauma. Where no clinical shock was present, the hormone levels were only just above normal. Where shock existed however, levels as

high as 60 µgms/100 mls. plasma were found. This is equivalent to the corticosteroid concentrations found after maximal adrenal stimulation by ACTH.

Mattingly and Tyler, 1965, investigated plasma corticosteroid concentrations during normal surgery. The induction of anaesthesia reduced the steroid level from 8-19 µgm/100 mls. to 1-5 µgm/100 mls. Surgical procedures however raised the concentration to between 25-50 µgms/100 mls. plasma depending on the duration and extent of the operation.

In case I the effect of adding 100 mgms of extrinsic hydrocortisone to a total blood volume of 5-6 litres is equivalent to a concentration of 2.0 and 1.66 mgms/ 100 mls. blood respectively. The concentrations found in the plasma varied from 1.0 - 3.6 mgms/100 mls. for the greater part of the operation.

Compared with cases 2,3 and 4 it is seen that the mean plasma corticosterone concentration of Case 1 is approximately 100 times too concentrated. It is reasonable to assume that under the conditions of Case 1 the adrenals virtually play no part in intrinsic corticosteroid secretion owing to the vast unphysiological excess of extrinsic hydrocortisone.

If it had been possible it would have been interesting to find out at what point the corticosteroid concentration "flattened out" i.e. reached physiological limits in this particular case.

CASE 2

The corticosteroid concentrations seen here are just above normal levels. The decrease seen in the first hour 11.45-12.45 is probably partly due to dilution of the blood during the first half of the perfusion. This dilution of the total blood volume was less than 10%.

It is possible that corticosteroid secretion during the middle phase of the operation did in fact decrease after the initial well marked response of the adrenals to surgical trauma - indicating somewhat less stress during this middle phase. This indeed would then be an absolute and not a relative change.

It would appear that adrenal cortical secretion was actively taking place during the 12.45-2 p.m. period. Haemodilution was negligible during this time.

CASE 3

This was a short perfusion of 30-45 minutes but it nevertheless shows an adrenal response of 18.0→25.5 µgms/100 mls. plasma.

CASE 4

This was a 3 hour perfusion. There was no noticeable increase in adrenal cortical output here.

Every effort is made to control the blood volume, fluids and electrolytes by replacing losses as they occur and even anticipating them. This is facilitated by most

precise monitoring. The apparent low 17 hydroxycorticosteroid levels may reflect the stability brought about by this great care and support observations on cardiac surgery mentioned by Walker 1965.

It would appear from the above results that the longer perfusions are not subjected to more stress (as measured by 17-OHC output) than the shorter ones, but that the adrenal cortical output of the former is obviously more prolonged due to the longer duration. In other words the total 17 OHCS output of long perfusions is due mainly to the duration of the operation rather than increased stress, as compared to the short bypasses.

The effect of dilution. An adult patient containing a blood volume of approximately 4 litres will when undergoing open cardiac surgery be connected with the Melrose heart lung machine containing 3 litres of perfusing fluid - 2 litres of whole blood (stored) and 1 litre of 5% dextrose or dextrose saline etc.

The addition of the latter isotonic solution to whole blood is equivalent to a dilution of 14% approximately.

Bearing this value in mind it is clearly seen from Figs. 2, 3 and 4 that the corrected corticosteroid concentrations would only be increased by some 2 - 5 µgms/100 mls. plasma. This increase would give final values that would still keep the adrenal response to open cardiac surgery in the mild to moderate category.

The above results agree well with those of Walker, 1965 and Hasner et al. 1962. The former found a rise in 17-hydroxycorticosteroids during perfusion of 9-25 µgms/100 mls. plasma. The increase in 17-OHCS in the total perfusing fluid amounted to 270 µgms/3 litres. This rate of adrenal secretion is not excessive when it is borne in mind that the rate of 17-OHCS in man has been variously estimated at between 28.0 and 64.7 µgm/min. by Hardy and Turner, 1957, and Hume and Bell, 1959, respectively.

In this work of plasma protein changes in cardiac surgery, 16 patients have been investigated over the last two and a half years. During this period the majority of patients have rapidly recovered and post operative shock has been a rarity.

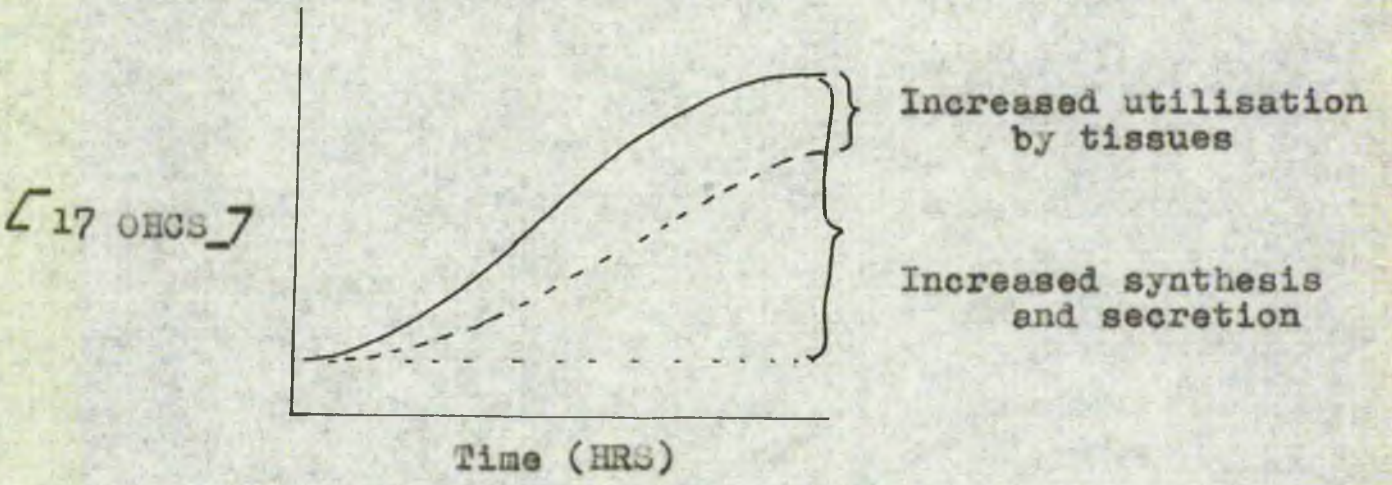
There is no one absolute indication that significant denaturation does take place during prolonged cardiac surgery from the results of the foregoing chapters. If, however, significant denaturation of plasma proteins did take place during perfusion it would not be unlikely to find 17 OHCS concentrations considerably raised. Einerth, 1965, found levels as high as 60 µgms/100 mls. in shock.

CONCLUSION

1) The addition of hydrocortisone (100 mgms 1 hrly.) during perfusion raises the mean plasma 17 OHCS concentration 100 times the normal values.

2) The 17 OHCS concentrations throughout perfusions in which hydrocortisone was witheld, showed only minor increases. The values obtained may appear low but could be explained in that increased utilisation of the hormone took place with coincident increased secretion, although not at the same rate.

It could be expressed thus :-



3) It would appear that prolonged cardiac surgery does not give rise to greater plasma 17 OHCS concentrations than are found in general surgery.

SECTION 10

Amino Acid Analysis of plasma and urine changes during and after extracorporeal circulation on full perfusion

INTRODUCTION

The purpose of this investigation was to find

1) Whether amino acids of plasma and urine, if possible, did increase during full perfusion at open heart surgery, and assuming this took place, study

2) The relationship of the increase versus time

3) The ratio of the individual amino acids to each other - and how this differed from the control.

4) The deleterious effect of this increase, on the renal system, leading to post operative complications and possible mortality.

5) The protective effect if any and if possible a clinical trial of L-Arginine HCl (GUILLINO) on the renal system.

All amino acids in sufficiently high concentration are hepatotoxic and nephrotoxic. Guillino et al. 1956, has shown by LD_{50} experiments on rats that the amino acids (L-form) differ quite considerably in the doses needed to cause death through toxicity. A list may therefore be drawn up showing the degree of toxicity of some of the common amino acids.

Isoleucine	ISOLEU	- Least toxic
Alloisoleucine	ISOLEU	↓
Valine	VAL	↓
Leucine	LEU	↓
Phenylalanine	Ø Al	↓
Methionine	METH	↓
Threonine	THR	↓
Allothreonine	A.THR	↓
Histidine HCl	HIST.HCl	↓
Lysine HCl	LYS.HCl	↓
Arginine HCl	ARG HCl	↓
Tryptophan HCl	TRYP HCl	Most toxic

The common amino acids not mentioned in this work (Guillino) are

Aspartic Acid	ASP
Serine	SER
Glutamic Acid	GLUT
Proline	PRO
Glycine	GLY
Alanine	ALA
Cystine	CYSS
Tyrosine	TYR
Hydroxylysine	OH LYS
Ornithine	ORN
("Ammonia")	NH_3

It seems remarkable that such a large number of common amino acids occurring in plasma and urine were not included in this work. Using LD_{50} data GUILLINO shows a sevenfold difference in toxicity between L-isoleucine and L-tryptophan.

There appears to be no common factor e.g. physical or chemical which can be proposed that explains this order of toxicity.

GUILLINO does not mention the concentrations of amino acids normally occurring in rat plasma. If one assumes that the ratios of the amino acids to each other is similar to humans then it is strange that he includes Methionine which is present in low concentration in human plasma 0.85 mgms/100 mls. (MEAN), and omits the common amino acids mentioned above.

It is important also to emphasise that the order of amino acid toxicities in rats may well not be the order of toxicity in humans.

It has been shown by Newburgh, 1925, Lewis, 1925, and Fishman, 1945, that the most toxic amino acids are

L - TRYPTOPHAN

TYROSINE

HISTIDINE

LYSINE

The L-forms normally occurring are more toxic than the D-forms.

It is pertinent to mention that Guillino's findings on the LD_{50} value of Arginine was next to tryptophan in toxicity, but that Arginine when added to a lethal mixture of nine essential amino acids decreased the mortality of the rats from 99.9 to 23%. This minimum mortality was reached when Arginine was present in a concentration of 1.5 mM per 100 mM of a mixture of nine essential amino acids.

These facts show very lucidly the vast importance of the effects of non physiological concentrations i.e. $L.D._{50}$ dose, and "physiological" concentrations on mammalian experiments. Arginine having a protective effect in small concentrations and a lethal effect in high concentrations.

Dimililer and Trout in 1965 showed that most of the plasma amino acids did increase in patients on cardiopulmonary bypass. This will be compared and commented on in the discussion of this section.

It is of great importance in amino acid studies to compare whenever possible the urinary changes concomitant with plasma changes. The essential amino acids are normally almost completely reabsorbed from the glomerular filtrate. The direct comparison of plasma and urine however is made confusing for the following reasons.

1) The renal clearances of amino acids differ by small factors.

2) Amino acid concentrations in plasma vary markedly from glutamine (mean 8.3 mgm/100 mls.) down to aspartic acid (mean 0.03 mgm/100 mls).

3) A significant increase of one or more amino acids in the plasma blocks or saturates the renal reabsorption of others, and consequently alters the urinary amino acid picture.

From 2) above the seemingly paradoxical situation arises between glycine and histidine. The latter has a higher renal clearance (10-5% of the glomerular filtrate) than glycine (5-3% of the G.F.), yet glycine occurs in higher concentration in the urine because of higher plasma values.

An example of 3) above is hereditary prolinuria (Scriver) in which an increase in plasma proline saturates a specific proximal tubular reabsorption system for not only proline but also hydroxyproline and glycine. Consequently all three amino acids are found in the urine. This is an example of an amino aciduria of the renal type as opposed to the more common, overflow threshold type, - which includes phenylketonuria, maple syrup urine disease, citrullinuria and histidinuria.

The overflow non threshold amino aciduria includes the rarer cystathioninuria, hypophosphatasia and arginino succinic aciduria.

Hereditary defects of amino acid transport confined to the proximal renal tubules result in another type of amino aciduria but is outside the scope of this work.

Urine collections during perfusion

The importance of concomitant collection of urines at the same time as plasma collection was the aim in all of the perfusions studied. However many practical difficulties made this objective virtually impossible to achieve for the following reasons.

1) In the vast majority of cases there was no urine flow at all during perfusion due to

a) Low aortic pressure resulting in minimal glomerular filtration

b) Blocked or kinked catheter.

20% Mannitol (40 mls.) one hourly was given in several cases, and was usually successful in producing urine - however small a volume.

2) In spite of constant supervision some urines got inadvertently thrown away.

3) Very often when there was urine flow during perfusion it was impossible to obtain regular fractions.

Perhaps the best that could be done would be to obtain an immediate preoperative specimen and a specimen at the end of perfusion.

4) It is of paramount importance that if urine collections are made during perfusion, then effort should be made to collect the whole 24 hour period, since urinary amino acid excretion patterns are best interpreted on a 24 hour basis.

The Amino Acid Analysis.

The amino acid analysis of both plasma and urine was done on a Phoenix Amino Acid Analyser and concurrently with this two way paper chromatography of amino acids was utilised.

1) To compare with the quantitative automated results.

2) To distinguish any abnormal spots and help the elucidation of initially unidentified peaks seen on the analyser.

In regard to urine results this latter fact was of great importance owing to variation of urinary amino acids from individual to individual in normal health. Patients on perfusion however show even greater urinary amino acid changes - which can still be looked upon as within physiological limits.

Additional amino acids looked for in urine not found in plasma were

1) β amino isobutyric acid (individual variation)
2) 1 - Methyl histidines
3) - " "
4) β Alanine.

On all urine specimens total nitrogens were done and correlated with the amino acid findings. The other parameters and factors obtained from plasma and urine aliquots at and after perfusion are mentioned in the section on perfusion.

The preparation of amino acids from plasma and urine is described. The technique of preparation for the Analyser is simpler and quicker than that required than when paper chromatography of the acids is to be done. This is due to one very important reason :-

Paper chromatography can only be successfully performed when the sample contains very little or preferably no inorganic salt (of any nature). Blurring of the spots due to poor separation occurs when salt is present probably due to the formation of strongly hydrophylic centres which extract water from the solvent to form pools of water on the paper. This obviously upsets the saturation and solvent flow equilibrium in the enclosed tank.

Desalting of samples can be done in three different ways.

1) Electrolytically - to be described in this series.

2) Column separation using cation exchange resins such as ZeoKarb 225 (Dowex 50, IRC 120).

3) Ultrafiltration through dialysis membrane.

The method of desalting depends on the apparatus available and the volume of sample to be desalted.

For paper chromatography where only a small volume is required the electrolytic procedure is the method of choice.

Using the column separation method by cation exchange resin is ideal for larger volumes (5-10 mls.) but has the disadvantage of 1) Diluting the starting sample, and 2) being time consuming.

3) Taurine is lost and arginine is retained on the column - the latter amino acid can be eluted however with stronger ammonia (3 $\overline{M}$) than is used for normal amino acid elution (2 $\overline{M}$).

Choice of deproteinising procedure/agent

There are well known, well documented procedures on deproteinising techniques.

Folin and Wu 1919 used sulphuric acid and tungstate. Haden, 1923, and Van Slyke et al. 1928 modified this technique.

Duggen and Udenfriend, 1956 used a tungstate technique and barium to precipitate the former as barium tungstate. The excess barium was in turn precipitated as the carbonate.

Somogyi, Shaffer and Hartman deproteinised haemolysed blood by zinc hydroxide.

Other methods include the use of trichloroacetic acid (T.C.A.), perchloric acid, and heavy metal salts (mercuric chloride, silver nitrate etc.).

It is of interest that Neuberg 1950 used a basic mixture of mercuric acetate and sodium carbonate as a selective precipitant for amino acids only, from a mixture of substances in a protein hydrolysate. All non amino acid material remains in solution.

All of the above materials use unphysiological ions which, if care is not taken to remove them might interfere with the final analysis when the sample is loaded on to the column (resin) of the amino acid analyser. In fact, the use of alkaline salts of heavy metals (mercury and zinc) does not only remove proteins but <u>also</u> some of the major non protein nitrogenous constituents of the blood such as

creatinine, uric acid, glutathione and methionine. Clearly this procedure is of no use in the determination of the N.P.N. of blood.

The method used in the amino acid separation from samples in the following work was simply the precipitation of proteins by the use of ethanol. This had the great advantage of being.

1) Simple and short, and
2) No foreign inorganic ions were added to the samples and
3) The use of alcohol facilitated the vacuum evaporation procedure which is necessary whatever preliminary method is used.

Although only 10 ml. aliquots of plasma and urine were used for this analysis, Westall, 1952, has used up to 18 Litres of urine to isolate amino acids and other ampholytes. It is of interest that he too has used ethanol in the initial precipitation procedure. Not only are no amino acids lost but all protein and most peptides (depending on the exact alcohol concentration) are precipitated together with many inorganic salts.

As mentioned previously all samples were desalted - after deproteinisation - when paper chromatography was employed.

METHODS

The extraction of free amino acids from plasma and urine

Normal urine contains no protein (or very little), but because albuminuria (usually slight) is common after stress - such as the trauma of operation - urine was treated by the same deproteinising procedures as was plasma.

In this study only the common amino acids were being analysed, for this reason the volume of 10 mls. for both plasma and urine was used as an adequate aliquot.

To 10 mls. plasma in a boiling tube was added 10 mls. 99% Ethanol : 1 N hydrochloric acid mixture (90.10 v/v). The contents were well mixed and immediately centrifuged.

The supernatants were poured into a 50 ml. round bottomed flask and evaporated to near dryness at 45-50°C. 15-20 mls. 85% Ethanol was added to the flask in increments, the contents dissolved, poured into two centrifuge tubes and again immediately centrifuged. In this way any protein or moderate sized peptides which escaped precipitation in the first alcohol extraction (45% approximate concentration) are precipitated in this higher concentration.

The supernatant(s) were again transferred to a clean round bottomed 50 ml. flask and evaporated to near dryness at the same temperature approximately - (the second evaporation).

The contents of the flask were gently dissolved in 1-2 mls. 10% isopropanol and the volume carefully made up to 3 mls. The exact final volume must be known for calculations of dilutions etc.

This sample was stored in a 5 ml. or equivalent test tube to which was added 1-2 drops of merthiolate to prevent bacterial decomposition.

The urine samples were treated in exactly the same way.

The extraction of total amino acids from plasma and urine

The method follows the above up to the end of the second evaporation. At this stage 10 mls. 6N Hydrochloric acid was added to the flask and the contents thoroughly dissolved. The mixture was poured into a clean hydrolysis tube, the screw cap of which contains a circular teflon patch.

The tube was left in an oven for 16 hours at 105-110°C.

Initially varying hydrolysis times of 16, 24 and 30 hours were compared for results. The 16 hour results showed virtually no difference to the 24 and 30 hour results. A 16 hour hydrolysis period was taken as the minimum time necessary to produce accurate and reproducible results.

As ethanol was used in the protein precipitation, some low molecular weight di and tertiary peptides (e.g. carnosine and glutathione) probably escape this procedure and remain in the supernatant. It is therefore only in the hydrolysis procedure that the constituent amino acids - alanine, histidine (carnosine) and glutamic acid, cysteine, glycine (glutathione) - of these peptides and most likely several others, are liberated.

Therefore the final concentration of liberated amino acids is the sum total of those derived from excretory detoxication products, urinary esters, products of hydroxylation, oxidation, reduction and conjugation etc. - as well as peptides mentioned above.

Apropos of the significance of hydrolysis times it is worth mentioning the concise survey of Tristram and Smith 1963 on the importance of this factor on the amino acid analysis of proteins.

Following hydrolysis the hydrolysate was centrifuged to get rid of any humin particles. The supernatant was then concentrated in vacuo at 45-50°C to approximately 1 ml. 10 mls. 85% Ethanol was added to the flask and the concentrate dissolved, this was then centrifuged and the supernatant containing the amino acids carefully re concentrated in a clean round bottomed flask (25 ml.). This was repeated twice.

Finally 2.5 mls. of 10% isopropanol was added to the concentrate to dissolve it and the volume carefully made up to exactly 3 ml. as mentioned in the free amino acid extraction 1-2 drops of merthiolate were similarly added. The concentrates were stored at 2-3°C.

Estimation of total nitrogen of urines/plasma

The total nitrogen procedure was used not only for the determination of protein or amino acid concentration, but also as an ancillary procedure to compare with quantitative amino acid results after and before hydrolysis i.e. Total and free amino acids.

The micro kjeldahl apparatus was used.

2-4 mls. of plasma, urine or equivalent sample (the exact volume must be known of course) were placed in a hydrolysis kjeldahl flask, 2 mls. of nitrogen free concentrated sulphuric acid was added carefully and 1/3 - 1/2 crushed tablet of kjeldahl catalyst (copper sulphate, sodium sulphate) was added. The latter - sodium sulphate may be used alone.

Plasma samples were left hydrolysing overnight. It is of interest that it is generally recognised that samples are left hydrolysing until the solution has cleared.

However it is common knowledge that in hydrolysis of urine only 30 minutes is suggested for this procedure - using a normal (control) urine. However if this time is adhered to one finds that the solution has indeed not cleared but remains dark orange colour.

Total nitrogens were therefore done on samples of normal urine after ½ hour and after 10 hours (when the solution was clear). There was no difference in total nitrogen content whatsoever between these two hydrolysis times.

As a safeguard post operative urines were hydrolysed for 10-16 hours. The overnight period is most convenient for this.

At least two estimations per sample were done. The blank consisted of water in lieu of the protein or urine sample.

Following the overnight digestion the flasks were allowed to cool and a few mls. of nitrogen free distilled water gently ran down the side of the flask neck and gently mixed.

The most accurate, consistent results are obtained when using the Kjeldahl distillation apparatus by the following method.

The apparatus is cleared of atmospheric ammonia and nitrogen by "steaming it through" for several minutes.

Then leaving the waste tap OPEN below the reaction chamber, the protein sample was washed in to the latter, 10-15 mls. of 30% sodium hydroxide was poured in (exact volume not critical).

The apparatus was steamed through for exactly 2½ minutes after switching the element on and closing the waste tap <u>simultaneously</u>.

The bottom of the condenser was kept just below the surface of the 2% boric acid (5 mls.) in the receiving flask (conical). The boric acid was made up thus :

TSIRO'S indicator.

50 mgms methyl red and 112 mgms of bromocresol green was made up to a volume of 62.5 mls. with absolute alcohol. This stock solution was kept tightly stoppered. It remains stable for many weeks.

10 mls. of this stock indicator was diluted to 250 mls. with 2% boric acid. This gives a darkish red solution. When ammonia is passed through this solution the colour change is very distinct and passes rapidly to a light green colour.

At the end of 2½ minutes the conical flask was lowered and the tip of the condenser washed with a few mls. of distilled water - nitrogen free and after a further ½ minute the flask was removed and corked with cotton wool.

Titration of the distillate was done with N/70 hydrochloric acid, 5 mls. of which is equivalent to 1 mgm. of nitrogen.

The end point is the change from the light green the distillate to a sudden pink. This is very abrupt and for this reason this indicator is thoroughly recommended.

The Standard Nitrogen solution

The standard nitrogen solution used originally to test the method was the following.

0.236 gms. of Analar ammonium sulphate was dissolved in several mls. of nitrogen free distilled water, a few drops of concentrated sulphuric acid added, and the volume made up to 1 Litre. It is essential that dry ammonium sulphate is weighed out precisely and to this end it was dried overnight in an oven at 100°C and cooled next morning before weighing.

This solution which is stable indefinitely contains 0.05 mgms. of nitrogen per ml.

These samples, - free and total amino acids - which were analysed on the 'Phoenix' were not desalted per se by the following electrolytic method, there being no necessity. However, it is worth mention that the effect of using 85% ethanol in extraction is one of precipitating most inorganic

salts - another advantage of the solvent's utilisation.

The following desalting procedure was used solely in the preliminary preparation of amino acids for paper chromatography.

Desalting technique: Electrolytic method

As mentioned before the method of choice of desalting small samples is that of electrolytic desalting. This has the advantage of being quick, reliable and giving rise to virtually no loss of the common amino acids. The apparatus used was the Electrolytic Desalter MK 3 and Power Pack of the Locarte Company, London (formerly 24 Emperors Gate, London, S.W.7).

There is well documented literature on this available from the makers.

Only a number of important points will be mentioned here, upon which success with the technique depends.

Principle

When the anode is in contact with the cathode via the sample to be desalted, and the current switched on, inorganic cations and anions travel to the cathode and anode and are washed away by the sulphuric acid and mercury (and water) respectively. If small enough, organic anions also are conducted through the membrane into the anodic effluent.

Organic cations deserve special mention since amino acids soluble in the original solution may become partially insoluble in the desalted version. Tyrosine, cystine, taurine and indole acids may suffer partial loss during the procedure. Although conversion of arginine to ornithine has been recorded by Stein and Moore, 1951, and Astrup et al. 1951 as well.

Apparatus

The electrolytic desalter is of vertical design with the lower circulating mercury pool (cathode) holding the sample e.g. 2-4 mls. in the centre compartment. The height of the mercury is solely controlled by water pressure from a nearby tap.

The upper electrode is the anode, the circulating electrolyte of which is 1-2% sulphuric acid which flows through the anode from a reservoir (3-5 Litres) situated above it.

The lower end of the anode has a viscap membrane securely attached to it by an elastic band and it is this which is kept just in contact with the surface of the sample to be desalted.

Both electrodes are connected with the Locarte Power Pack which gives a voltage of 200-250 D.C. with a current range of 0 - 2 amps.

A similar apparatus to the above is the electrodialyser which consists of a central compartment - containing the sample - which is separated from both electrodes by a semi-permeable membrane. This may easily be made from perspex sheets.

Method

Although the desalting procedure itself causes protein denaturation, plasma was deproteinised by ethanol prior to desalting for paper chromatography. Urine need not be "deproteinised" beforehand, although it is advantageous, and prevents any frothing which occurs quite commonly otherwise. This frothing may be reduced by a few drops of acetone, but it is an annoying feature.

Although the literature from Locarte suggests an anodic electrolyte concentration of 2%, it was found that in the absence of a rheostat this concentration is far too great - the reaction proceeding too vigorously and exacerbating any frothing which might take place. Much heat is produced and its dissipation from the site is dependent on the flow of acid through the anode. 50-100 mls. a minute is a good rate when a large quantity of heat is being produced. The temperature of the effluent acid rarely exceeds 27°C.

When the power pack is switched on the current rises to between 1-2 amps. As desalting continues the current gradually falls to a level at which it remains.

This final current value differs according to the type and concentration of the sample. At this point the sample is virtually desalted.

The rate of desalting may be accomplished in two ways :

1) by a suitable rheostat.

2) by adjusting the anodic electrolyte concentration.

The more concentrated this is, the quicker is the desalting process and the greater the heat produced. The latter is counteracted by speeding up the rate of flow from the reservoir. A great disadvantage of having too fast a rate of desalting, is that the Viscap membrane gives way and dilute sulphuric acid begins flooding the sample chamber. Therefore each run must have a new membrane filled before desalting starts.

It was found that the best anodic electrolyte (sulphuric acid) concentration was 0.25 - 0.5% - the exact concentration is not critical. Using this solution of low conduction prolongs the desalting process to roughly 20-30 minutes.

If 2% acid is used the desalting time is cut to between 3-5 minutes.

As the desalting time is short no change or loss occurs in the amino acid content of the sample. In longer desalting times however tyrosine, cystine, taurine and the indole acids may undergo partial loss. The possible conversion of arginine to ornithine has not been satisfactorily proven, probably due to widely differing apparatus and experimental conditions. Certainly in the short desalting time here arginine showed no quantitative loss. Samples were analysed on "the Phoenix" before and after electrolytic desalting and the results of tyrosine and arginine particularly examined.

Paper chromatography

Introduction

Amino acids in the samples were analysed by sometimes one way and always two way chromatographic techniques. Ninhydrin staining was supplemented with the multiple dip technique to aid identification. The results obtained were compared with the quantitative results from "the Phoenix" Analyser.

Method

Chromatography papers No. I, 4 and 54 were all used for amino acid separation with excellent and similar results.

Prior to paper chromatography an amino acid sample was reconcentrated and taken up to the same volume with 75-80% Ethanol in order to facilitate the application to the paper to give a discrete, small concentrated starting spot.

25-50 µl. of amino acid concentrate equivalent to 0.08 - 0.16 mls. plasma or urine were spotted on the paper.

The solvent systems were :

Butanol-Acetic Acid-Water (BuAc) 12 : 3 : 5.

This was always used first, followed by :

Phenol-Ammonia (.880) (Ph. Am) 200 : 0.5.

Bu Ac was used only twice and on consecutive nights. The length of run was 16 - 18 hours.

The second solvent mixture Ph. Am. was made up by mixing 160 gms. phenol with 40 mls. water. This may be left overnight to form a homogeneous solution or it may be gently warmed to facilitate this.

Although 1 ml. of 880 Ammonia is recommended to 200 mls. Phenol, it was found that it causes considerable darkening at the solvent front and some background staining after a 14-18 hour run. Therefore the volume of ammonia added was reduced to 0.5 mls. per 200 mls. phenol. It is important that the ammonia is added only immediately before the run is to take place. Its value is that it increases the R_f values of the basic amino acids thus giving better separation.

Immediately following the phenol run the chromatogram was dried for several hours in a fume cupboard - in the COLD. If heating was used and spots appeared which did not arise in the cold (room temperature) then they would most unlikely be amino acids.

Staining with ninhydrin was done either by spray or by the dipping technique (0.2 gms ninhydrin/100 mls. acetone). All the spots were seen in 30 minutes - 2 hours. If the chromatogram was not to be used for the multiple dip

technique it was preserved by dipping through a solution of 0.5% nickel sulphate in water.

In one of the accompanying photographs a chromatogram is shown having been stained with Ehrlich reagent. The amino acids appear quickly, at different rates and with differing intensity. These were noted and shown on an accompanying diagram. It is important to note these changes since many of the spots fade away and reappear later. Most amino acids have their own mode of presentation, indoles (tryptophan) are purple and most of the other amino acids are blue or yellow.

<u>The multiple dip sequence</u>

Initially Ninhydrin stained chromatograms were treated thus :

Nitrosonapthol was used to detect tyrosine (red) and indoles (grey or brown).

Sulphanilic acid was used to detect histidine and other imidazoles, tyrosine and other phenols. Brown, yellow or red spots form if these amino acids are present.

Anisidine reagent is similar to sulphanilic acid in that it detects histidine and the other imidazoles (red and brown), tyrosine (light brown).

For make up of reagents see Appendix I.

With the two latter reagents the chromatograms were first washed with ether to elute the remaining phenol, since they react very poorly in its presence.

Since no unknown amino acids were detected either chromatographically or by the analyser, most chromatograms were just stained with ninhydrin with the occasional supplementary Ehrlichs reagent. Ninhydrin and Ehrlichs reagents stain all the amino acids whereas nitrosonapthol, sulphanilic acid and anisidine are very much more selective in the acids detected.

Hydroxyproline analysis

Mention must be made of the slight difficulty in the elucidation of the first few peaks of amino acids as they occur on the Phoenix analyser. These are taurine, cysteic acid, urea and the elucidation of a tall peak which was quantitated on the 440 mμ wavelength - i.e. the same as proline. Since 3 and 4 hydroxyproline are eluted very

early from the column it appeared reasonable to do a direct hydroxyproline analysis. This was done modifying Stegmans method.

0.2 mls. of a plasma amino acid hydrolysed concentrate equivalent to 0.668 mls. plasma was taken as an aliquot for this trial estimation. This 0.668 mls. plasma is equivalent to over 250 mgms. of amino acids per 100 mls. plasma and was equivalent to the volume and concentration of sample used on the analyser.

Reagents 1) Chloramine T. solution.

2.82 gms of chloramine T. (p. toluene sulphanyl chloride sodium salt) was dissolved in 40 mls. distilled water, 60 mls. of methyl glycol and 100 mls. of acetate citrate buffer, pH 6.

2) 4 M Perchloric Acid 160 mls. 60% was diluted with 210 mls. water (dist.).

3) A 10% p. dimethylamino benzaldehyde solution was made in methyl cellulose.

Method

The 0.2 mls. of concentrate was diluted with acetate citrate, buffer pH 6, to 2 mls. and added to 1 ml. of the chloramine T. solution, shaken and left for 20 minutes at room temperature. 2 mls. of perchloric acid was then added,

the solutions shaken and left for 5-15 minutes. 1 ml. of p. dimethylaminobenzaldehyde was added with shaking and the resulting solution heated to 60°C in a water bath for 15 minutes.

After cooling the absorption was read at 557 mμ.

No absorption was noted and thus hydroxyproline was considered to be either absent or in very low concentration. It is interesting to note that hydroxyproline has been demonstrated in animal sera (Documenta Geigy 5th Ed.) but not so far as the author is aware in man.

Plasma and urinary amino acid results from open cardiac surgery

The quantitative amino acid results obtained from the Phoenix Analyser were converted from micromoles to

1) mgms/100 mls. for plasma

and 2) mgms/24 hrs. for urine.

The pattern of plasma and urinary amino acids of 17 patients during and following open heart surgery were investigated. The time on full perfusion varied from 30 minutes to over 3 hours. The longest was Ch. AVR 68.3 which lasted 4–4½ hours.

The patients were of widely differing ages, 5–65 years, and of differing cardiac lesions, e.g. Aortic and mitral valvular defects, atrial and ventricular septal defects.

The difficulty in elucidating the exact amino acid changes are due to the following varying factors.

1) Varying perfusion times

2) Varying age and sex and apropos of this - surface area, weight, and metabolic rate.

3) However constant operational and ancillary techniques are kept, it is obvious that minimal small

changes will at least occur, e.g. time of addition of blood, saline dextrose, bicarbonate, mannitol, drugs etc. mentioned in the Introduction Section I on Perfusion technique.

Some post operative urine results were unable to be obtained due to several reasons, the most important being :-

1) Fallability of nursing routine.

2) Patients decease.

The amino acid results of plasma have been analysed

1) by the comparison of control, and final plasma concentrations in the form of histograms. These histograms of each operation are seen in Figs. 10.1 - 10.17.

2) by dividing the 17 cases into short (½ - 1 hr.), medium (2 hrs.) and long (3 3½ hrs.), and illustrating the increase of individual amino acids with time, thus :

30-60 mins.	2 hrs.	3-3½ hrs.
6 Cases	3 Cases	8 Cases

Mean values of plasma amino acid concentrations were derived using the amino acid values taken at 30-60 minute intervals at each operation.

The 45-60 minute mean value of each amino acid was derived from 13 operations.

The 90-120 minute mean value of each amino acid was derived from 7 operations and the 150-180 minute mean value derived from 6 operations. The decrease in the number of operations used (to obtain the mean value) with increase in time are due to two factors.

1) A number of operations (5) were of only 30-60 minutes duration (excluding the control "17 D.M.") and 3 were of moderate duration - 120 minutes.

2) (a) One operation "I.J.M." had 21 pints of blood added during the 3½ hour operation. Although the results are recorded they were not used in the elucidation of the mean values.

(b) The operation "15 McC" was a 3 hour control operation and these values were similarly omitted and recorded separately.

Where 30 as well as 60 minute values have been obtained the latter only were used to derive the mean values.

The increase in individual plasma amino acids with time of perfusion are seen in Figs. 10.18 - 10.23. The maximum and minimum results are also recorded.

Two of the operations were analysed as "controls".

One (D.M.) was an anterior resection of colon for carcinoma which was a 30-45 minute procedure, the other (J.M.) was a carcinoma of the oesophagus complicated by a hiatus hernia, for which a resection was performed as well.

As many amino acids were analysed as possible. In plasma all the common amino acids are easily identified above concentrations of approximately 0.2 mgms/100 mls. (β alanine and β amino isobutyric acid B.A.I.B.). Two or three amino acids in plasma were not able to be quantitated as standards were not available, but where they have occurred, mention is made.

In the urine however, striking changes can and do occur even in normal individuals (e.g. the excretion of B.A.I.B., peculiar to some individuals). In pathological conditions, pregnancy and stress (e.g. operations) the amino acid pattern is greatly altered.

Below is a Table 10.1 comparing the mean control values of plasma and urinary amino acids (using the method described in this Section) with those values found in the literature.

A = Mean Total, free (non protein) amino acids of plasma compiled from numerous references mentioned in Documenta Geigy 5th Ed.

B = Mean Amino acids of plasma based on data of Stein, 1953, Stein Bearn and Moore, 1954, Stein and Moore, 1954, Evered, 1954, and Archibald, 1944.

C = Mean amino acid concentrations of pre-operative control plasma samples after acid hydrolysis (this study).

A' = Mean total amino acid concentration in normal urine after Hier, 1948.

B' = Mean amino acid concentrations (free) of normal urine derived from references seen in B above.

C' = Mean amino acid concentrations (total) of urines taken from surgical patients (pre op.), after acid hydrolysis, (this study).

Table 10·1

Mean control values of total amino acids in Plasma and Urine

	Plasma mgms/100mls.			Urine mgms/24hrs.			Abbreviations
	A	B	C	A'	B'	C'	
Alanine	3·97	3·41	2·79		46	183·0	Ala.
β Alanine		0·2	n/a			n/a	B.Al.
B.A.I B.			n/a			n/a	B.A.I.B.
Arginine	2·24	1·51	3·0	23·7±7·9		35·5	Arg.
Aspartic Acid	1·05	0·03	1·93	164·5±46·7	<10	115·0	Asp.
Aminobutyric Acid		0·3	n/a		<10	n/a	A.B.A.
Citrulline	0·5	0·5	n/a			n/a	Cit.
Cystine (½)	1·4	S-S and -SH 1·18	-SH 1·14	—	S-S and -SH 10	-SH 15·8	Cyst and Cyss
Glutamine	5·78	8·3	-			-	Glut. NH_2
Glutamic Acid	3·41	0·7	2·7	351·4±151·4	<10	320·0	Glut.
Glycine	1·77	1·54	2·11		132	319·0	Gly.
Histidine	1·42	1·15	1·64	203·3±101·1	216	129·8	Hist.
Hydroxylysine			0·35			"11.0"	OH Lys
Isoleucine	1·60	0·89	1·31	20·3±5·5	18	15·5	Isol.
Leucine	1·91	1·69	4·45	21·2±6·6	14	30·0	Leu.
Lysine	2·95	2·72	3·76	73·2±29·4		52·0	Lys.
Methionine	0·85	0·38	Tr—1·06 (0·59)	8·6±2·8	<10	32·2	Meth.
1. Me. Hist		0·11	n/a		180	n/a	1. Me. Hist
3. Me. Hist		0·08	n/a		50	n/a	3. Me Hist
Ornithine		0·72	1·58		<10	1·57	Orn.
Phenylalanine	1·38	0·84	1·69	23·3±7·9	18	27·2	Ø Al.
Proline	2·55	2·36	2·31	42·8±12·9	<10	51·5	Pro.
Serine		1·12	1·67		43	49·75	Ser.
Taurine		0·55	-		156		Tau.
Threonine	2·2	1·39	1·47	53·8±19·5	28	36·5	Thr.
Tryptophan	1·08	1·11	n/a	41·4±17·5	—	n/a	Tryp.
Tyrosine	1·48	1·03	0·91	52·5±18·0	35	30·3	Tyr.
Valine	2·83	2·88	2·88	19·8±5·6	<10	34·45	Val.

Discussion — The Amino Acid Changes in Plasma

Introduction

Due to the inter-related and complex changes of the amino acids of plasma and urine during perfusion any form of discussion has necessarily got to be a compromise in grouping between changes found, the metabolic rate of the individual acids and apropos of the latter the natural grouping of acids owing to their metabolic function.

It is right however to initiate this discussion with the results of the Melrose experiment.

The Melrose experiment

This control study was set up as described in Section I and serial samplies taken at ½ - 1 hourly intervals for 6 hours. No significant increase in plasma amino acids occurred throughout this period. The quantitative results are seen in Appendix II. The control values are well within the normal range. The final results show an increase from virtually nil to approximately twice the control values only.

This shows that the increase in amino acids seen in perfusions is not due to the in vitro destruction of

blood by oxygenation. It also shows that there is no significant association between the degree of haemolysis and amino acid concentration of plasma since the former showed profound haemolytic changes during the 6 hour experiment, (Section 5).

The results of the other criteria examined in these blood samples are seen in the appropriate sections.

The changes of plasma amino acids of patients on full perfusion

It is seen from the figures 10.2 - 10.23 that there is an increase in all plasma amino acids and this is related to the length of perfusion. The greatest increases are 1) in the first hour of perfusion and 2) approximately after 2¼ - 2½ hours when the curve again ascends more rapidly.

This is even more pronounced in perfusions of over 3 hours e.g. "4 Ch", "7 Mor," and "10 Moy".

Most of the amino acids follow a sigmoidal curve therefore with the possible exceptions of methionine, isoleucine, tyrosine, hydroxylysine, ornithine and histidine. These follow either a linear increase or after an initial rise reach an apparent "status quo".

These plasma amino acid changes may be classed into three groups for convenience.

1) Severe changes
2) Moderate changes
3) Slight or no change

1) Severe changes :-

Aspartic acid, glutamic acid, leucine and lysine.

2) Moderate changes :-

Threonine, serine, proline, glycine, alanine, valine, tyrosine, phenylalanine, histidine, arginine and "ammonia".

3) Slight or no change :-

Cysteine, isoleucine, methionine, hydroxylysine, ornithine and tryptophan.

As these amino acid changes are a function of their metabolic importance they will be discussed either in groups or singly according to their structure and/or participation in metabolism, rather than on their decreasing concentrations in plasma given above.

The urinary amino acid changes will be discussed with its plasma counterpart.

They are therefore discussed in the following order.

Aspartic and Glutamic Acids
Valine, leucine and isoleucine
Lysine
Threonine
Serine
Glycine
Alanine
Phenylalamine and Tyrosine
Proline, hydroxyproline, ornithine and arginine
Histidine
Methionine
Cysteine and Cystine
Tryptophan.

The Urinary post operative amino acids. Changes :

From the results of Stein, 1953 it has been shown that normally approximately 1.1 gms. of free amino acids are excreted in the urine per 24 hours. This is equivalent to about 180 mgms of nitrogen, = 1.2% of the total nitrogen excreted, = 120 mgms. of - amino nitrogen. After acid hydrolysis as much as 2 gms. - hitherto conjugated - of amino acids are also liberated. It is important to emphasise that these are only average values in normal urine. These figures become even more widely altered in conditions of varying amino acidurias with usually a concomitant plasma increase.

In urine the total amino acid nitrogen expressed as a percentage of total nitrogen is 4.0%. The free amino

acid nitrogen is correspondingly 0.8%. Urea is responsible for most of the urinary nitrogen - 84%. These results are Everetts, 1946.

Only five post operative urine studies were able to be investigated for the following reasons :-

1) Patients decease

2) Fallability of nursing routine

3) Logistic reasons of analysis.

Three post operative (perfusion) patients are compared with two post operative patients recovering from normal Thoraco-abdominal surgery. The results of individual cases are seen in Tables 10.25 to 10.43.

Other amino acids occurred in moderate concentrations in the urines. These were alanine, 1 and 3 methyl histidines. All three were detected by both analyser and paper chromatography techniques. As standards were not available, accurate quantitation was not possible. All three however were always present in pre operative urine samples, and had approximately similar concentrations in post operative specimens as judged by "peak measurements" (analyser) and density of spots (chromatography).

These amino acids may well have been derived from carnosine and anserine catabolism.

Owing to the great variation in urinary amino acid excretion in post operative patients, and the fact that

maximum excretion occurs at anything between the first and sixth post operative days, and that subsidiary peaks occur as well - the use of mean values has been discarded and the actual amino acid results are graphed from three patients in Figs. 10.25 - 10.42. An additional advantage of this mode of presentation is that absolute results of one amino acid from one operation may be compared directly with the results from the other operations.

These three patients ("3.7" and "15") were grouped because all three were operations of 3-3½ hours. Two ("3" and "7") were perfusion patients and "15" was the thoraco-abdominal operation acting as control. The other two patients, "8" and "14" in which post operative urines were obtained were short perfusions of 30-45 minutes.

Urine volume during perfusion

The urine secretion during perfusion varied enormously from total absence to over 100 mls. This was not dependent on duration. Since total anuria was not uncommon, mannitol was used I.V. to aid urine excretion, with some success. When urine was obtained it was occasionally inadvertently discarded. The mean volume collected during perfusion was of the order of 20-40 mls.

The discussion of both plasma and urinary results of each amino acid will be done together. The term "control" in the following discussion refers to the non cardiac operations.

The discussion of both plasma and urinary amino acid results during the operative and post operative periods.

Plasma Aspartic and Glutamic acids. See figs. 10.18 and 10.19.

Due to the fact that the deproteinised plasma samples were hydrolysed - thus releasing the higherto bound forms of aspartate and glutamate - it is not surprising to see a large increase of these two amino acids.

The mean values with maxima and minima of both acids are seen in the following table.

Aspartic Acid mgms/100 mls.

	Control.	1 hr.	2 hr.	3 hr.
Mean	1.93	12.64	15.64	25.32
Maximum		27.5	33.7	45.7
Minimum		8.73	9.4	10.92

Glutamic Acid

	Control	1 hr.	2 hr.	3 hr.
Mean	2.7	27.93	35.75	47.7
Maximum		39.67	61.2	84.3
Minimum		12.6	21.9	24.0

Both these non essential acids are active participants in deamination via transamination reactions. The conversion of ornithine to citrulline is aided by acetyl glutamate, and

aspartic acid aids similarly in the citrulline→arginine conversion. Glutamine (from glutamic acid) is the source of urinary ammonia. It is very interesting that the brain contains such aspartic acid as N-acetyl-L-aspartic acid. In the perfusions lasting more than 3 hours "4", "7" and "10" the aspartic acid levels were 45.7 32.5 and 36.1 mgms/100 mls. respectively. These concentrations are greatly above normal and it is pertinent to mention that patients "4. Ch." had a difficult prolonged (unconscious) post operative recovery due to apparent pulmonary and ? cerebral embolus. Patient "10 Mo" died post operatively from pulmonary infection, (10th day post op). Brief mention of the patients, perfusion data and comments are seen in Appendix II.

The glutamic acid levels were also very high in these "3 hour plus" perfusions. This amino acid too plays a very significant part in brain metabolism. Although glutamic acid is impermeable to the B.B.B. (blood brain barrier) its amine is freely permeable.

<u>The urinary results of aspartic and glutamic acids</u>

Figs. 10.25, 28.

As seen in Fig. 10.25 and 10.28 large post operative increases occur in the urine as would be expected from the above plasma changes.

Aspartic acid

As seen in the majority of urinary amino acids in the post operative period - two peaks are seen in the two perfusion cases. In the control operation only one broad peak is seen, although if the 6th and 7th post operative 24 hour urines had been available - a second peak could quite easily have occurred. The urinary aspartic acid concentration of the control operation (15 McC) mimics virtually the same degree of response as the perfusion cases. Although the longest perfusion case "4 Ch" (3½-3¾ hours) shows the smallest urinary concentration in the first peak 281.4 mgms/ 24 hours it makes up for this by having 3 separate peaks of diminishing concentration on days 2, 4 and 6 post operatively.

Glutamic acid

Again maximum excretion is seen on the 2nd post op. day. The control operation shows virtually the same concentration as in "7 Mor", although only exhibiting one peak.

The 3¾ hour "4 Ch" operation shows small excretion initially but reaches 800 mgm/24 hour levels on the 6th day.

Valine, Leucine and Isoleucine See Figs. 10.20 and 10.21

The mean, maxima and minima values of these acids are seen in the table below.

Valine

	Control.	1 hr.	2 hr.	3 hr.
Mean	2.28	10.38	14.79	24.99
Max.		14.32	32.6	38.5
Min.		1.2	8.9	12.2

Leucine (ditto)

Mean	4.45	23.89	28.51	43.88
Max.		42.0	40.27	86.4
Min.		12.9	20.1	18.0

Isoleucine (ditto)

Mean	1.31	1.88	2.80	5.36
Max.		4.6	5.82	7.0
Min.		0.3	1.25	0.44

These three essential branch chain aliphatic amino acids are discussed together as they have common metabolic features. From the perfusion results leucine is in the category of severe change, valine - moderate change and isoleucine in the slight change group. In the reversible transamination reactions which all these acids exhibit, the corresponding keto acids may replace the

respective essential amino acids in growing mammals. It is interesting that these three amino acids all occur in greatly increased concentrations in the plasma and urine of Maple Syrup disease in which the enzyme necessary for oxidative decarboxylation of branched chain amino acids is absent.

After the formation of the corresponding keto acids, the latter may be reversibly reduced to the hydroxy acids or degraded irreversibly in oxidative decarboxylation. In the diabetic animal the isopropyl group ($CH_3.CH.CH_3$) of valine is responsible for the donation of three carbon atoms in the formation of glucose.

Leucine follows a similar metabolic course terminating in acetyl CoA and acetic acid. It is the most Ketogenic amino acid.

Isoleucine which changed only minimally during perfusion follows a similar metabolic pathway as Leucine and Valine, forming glucose and acetoacetic acid. Three carbon atoms of isoleucine are responsible for the former and two carbon atoms for acetyl Co A formation.

It is not surprising therefore that in diabetic coma both leucine and isoleucine are increased in plasma and urine. All three amino acids are increased in muscular dystrophies.

Leucine concentrations in the three longest perfusions reached plasma levels of 61.3, 77.6 and 86.4 mgms/100 mls. in operations 3, 7 and 10.

The urinary changes of Valine, Leucine and isoleucine. Figs. 10.32, 36, 35.

Valine Both perfusion cases show double excretion peaks of high concentration. The control operation shows only one peak at a lower level of 207 mgms/24 hr. on the 3rd day.

Leucine Here again the control operation shows much lower excretory values than the perfusion cases. It does however show a double peak in common with these cases.

Isoleucine Lower levels are seen in the control than in the perfusions and only one peak is seen in the former, compared with two in the perfusions.

LYSINE See Fig. 10.23.

The mean, maxima and minima values of lysine are seen below.

	Control	1 Hr.	2 Hr.	3 Hr. +
Mean	3.76	20.33	27.06	47.3
Max.		33.54	52.7	99.0
Min.		10.96	12.4	17.6

This essential amino acid was the fourth to show severe changes, during perfusion. It is one of the most toxic mentioned by Guillino et al. Lysine is metabolised by initial irreversible deamination, the formation of a cyclic structure (piperidine carboxylic acid), and the successive reduction-oxidative - hydration steps form adipic acid derivatives. ∝ amino adipic acid does occur in urine and it was looked for in plasma and urine using chromatographic procedures. No unidentified peaks occurred in the amino acid analyser results. It was assumed therefore that if present in plasma (or urine) it was in concentrations of less than 0.2 mgms/100 mls. It is not a common constituent of plasma however.

∝ amino adipic acid forms via deamination, decarboxylation and oxidation, ∝ ketoglutaric and L-glutamic acids.

Lysine is increased in the blood and urine during diabetic coma. It is also regularly present in urine and blood during ACTH therapy, this will be commented on in the final discussion.

<u>The post operative urine changes of lysine</u>. Fig. 10.40.

Only one peak occurs in each of the 3 cases and the control reaches maximum levels of over 400 mgms/24 hours on the 4-5th days post op.

THREONINE See Fig. 10.18.

The mean, maxima and minima values of threonine are thus.

	Control	1 Hr.	2 Hr.	3 Hr. +
Mean	1.47	9.24	10.41	15.25
Max.		17.2	21.33	28.0
Min.		4.75	7.2	4.31

This essential amino acid comes in the moderate changes category. It undergoes deamination but not transamination. In the presence of pyridoxal phosphate it is degradated to glycine and acetaldehyde. Threonine also forms - after removal of the elements of water and oxidation, α keto and α amino butyric acids. The latter acid was also looked for in plasma samples and was occasionally found - but it was an inconstant feature of low concentration. α Keto butyric acid forms glucose upon oxidation.

An increase in plasma threonine also occurs in muscular dystrophy and in the urine of pregnancy.

Post operative urine changes of threonine. Fig. 10.26

The control shows similar concentration values to the perfusion results. Two post operative maxima are seen in the perfusion cases, and one peak in the control.

SERINE See Fig. 10.18.

The mean, maxima and minima values of serine are thus :-

	Control	1 Hr.	2 Hr.	3 Hr.
Mean	1.67	7.98	8.52	13.12
Max.		13.28	16.7	22.8
Min.		3.35	5.37	4.1

This non essential amino acid showed moderate changes in plasma concentration of the usual sigmoidal type. Serine and glycine are freely interconvertible in the body. L-serine dehydrase (liver) with pyridoxal phosphate deaminates serine to form pyruvic acid. In a transamination reaction with the latter, serine forms alanine and hydroxypyruvic acid. Serine is also a constituent of the phospholipids. Decarboxylation of serine produces ethanolamine. This amine was present occasionally in plasma in barely detectable amounts. This is the precursor of choline. Serine is also involved in the synthesis of cysteine.

Post operative urinary serine excretion. Fig. 10.27

All three cases show a similar rise to maximum excretion and as above the perfusion cases show two peaks whereas the control shows only one.

Glycine see Fig. 10.19.

The mean maxima and minima values of glycine are thus :-

	Control	1 Hr.	2 Hr.	3 Hr.
Mean	2.11	7.66	7.77	12.31
Max.		17.63	12.46	16.5
Min.		4.8	5.55	6.86

This amino acid increased only moderately in concentration and the mean value was reasonably constant during the 1-2 hour part of perfusions. This is perhaps surprising since hydrolysis procedure would be thought to liberate previously bound glycine. The amino acid plays a vast part in metabolism upon which there is much literature. Only a brief precis will be given here.

The conversion of glycine to glucose via serine has already been mentioned. It contributes its carbon to the one carbon pool as formate. It is possible that oxidation of methyl groups in the body to form formaldehyde aids the synthesis of serine from glycine. In this latter process requiring pyridoxal phosphate, GSH, FH_4 (tetra hydrofolic acid), TPN/DPN and Mg^{++} N^{10} hydroxymethyl tetrafolic acid (FH_4 CH_2.OH) is formed in the process and acts as a "carrier" for active one carbon groups. Finally it is concerned in

the synthesis of widely differing compounds such as glycocholic acid, glutathione (GSH), creatine, haem, glucose, purines and protein.

It would appear reasonable to hypothesise that the reason for only a relatively small increase in glycine during perfusions, may well be due to diminished hepatic function i.e. diminished enzyme activity which in turn limits the rate of glycine turnover - i.e. formation of and synthesis from glycine. This may well apply to other amino acids as well, although glycine is in a class on its own from the point of view of the vast complex of reactions it participates in. This will be discussed further in the Final Summary.

Post operative urinary glycine excretion. Fig. 10.30.

These results show marked changes which are consistent with a large increase in free glycine after hydrolysis. There is a marked fall in glycine concentration in the immediate post operative period but a maximum is quickly reached of 865 mgms/24 hours by the 3rd day, "4 Ch.". The other perfusion case showed less dramatic but still well marked changes. The control operation showed the least change - but even here an increase of 200 mgms/ 24 hours is seen over the pre-operative concentration. Two peaks are seen in all three cases.

ALANINE See Fig. 10.20.

Mean, maxima, and minima values of alanine

	Control.	1 Hr.	2 Hr.	3 Hr.
Mean	2.79	12.32	15.72	26.44
Max.		22.0	29.9	41.1
Min.		8.94	10.97	12.85

This non essential amino acid increased at concentrations in "the moderate range". It is one of the few amino acids which is relatively unimportant from the point of view of having few specific functions in metabolism. Deamination to pyruvic acid and ammonia by L-amino oxidase, and transamination with ketoglutaric acid to give pyruvic acid and L-glutamic acid, both occur. As with most of the foregoing amino acids the most dramatic changes are in the first hour and after 2½ hours of perfusion.

Post operative urinary alanine excretion. Fig. 10.31

A large increase in alanine occurred in all three cases with the control case giving the least values of all - about 60% of the perfusion values. All three showed double excretory peaks.

PHENYLALANINE AND TYROSINE See Figs. 10.21 and 10.22

The mean, maxima and minima values

PHENYLALANINE

	Control	1 Hr.	2 Hr.	3 Hr.
Mean	1.69	11.22	13.42	20.56
Max.		21.4	32.6	37.34
Min.		7.8	8.5	9.04

TYROSINE

	Control	1 Hr.	2 Hr.	3 Hr.
Mean	0.91	5.29	6.3	9.03
Max.		9.0	15.78	18.17
Min.		3.33	3.15	3.3.

These two amino acids are well known owing to their participation in the inborn error of metabolism - phenylketonuria, and related errors from the metabolic pathway point of view, such as albinism, tyrosinosis and alkaptonuria.

The acids are also important in the synthesis of adrenaline, noradrenaline, thyroxine, DOPA and melanin. Phenylalanine is an essential amino acid whereas its irreversible

hydroxylated derivative tyrosine, is non essential.

The end products of the tyrosine metabolic pathway are fumaric acid - which enters the T.C.A. cycle - and acetoacetic acid which is converted to Acetyl Co A.

Both these amino acids show a sigmoidal shaped increase during perfusion.

The conversion of phenylalanine to tyrosine depends on two enzymes phenylalanine oxidase I and II, molecular oxygen, pteridine coenzyme and reduced NADP. This is a complex reaction.

The conversion of tyrosine to p. hydroxy phenylpyruvic acid depends on another hepatic enzyme - tyrosine αketoglutaric transaminase.

<u>The post operative urinary phenylalanine excretion.</u>

Fig. 10.37.

Here there is marked contrast between the perfusion results and the control. The former rises from a pre operative level of approximately 25 mgms/24 hours to between 430 and 567 mgms/24 hours, whereas the control - although it definitely increases does so by only 75 mgms/24 hours.

<u>The post operative urinary tyrosine excretion.</u> Fig. 10.37

It is interesting that the changes seen with phenylalanine are mimicked by tyrosine - but at a lower

level. All three show two peaks. The control is at a substantially lower level than the perfusion results.

PROLINE, (HYDROXYPROLINE) ORNITHINE AND ARGININE

Figs. 10.9, 10.22 and 10.23

As mentioned above some amino acids are best mentioned together as their metabolic roles are so closely related.

PROLINE : Fig. 10.9.

	Control.	1 Hr.	2 Hr.	3 Hr.
Mean	2.31	7.13	8.45	14.78
Max.		13.6	17.5	22.5
Min.		4.73	5.1	7.55

ORNITHINE : Fig. 10.22

	Control	1 Hr.	2 Hr.	3 Hr.
Mean	1.58	0.98	0.805	1.024
Max.		1.79	1.33	1.41
Min.		0.44	0.5	0.71

ARGININE : Fig. 10.23

	Control	1 Hr.	2 Hr.	3 Hr.
Mean	3.0	7.73	8.85	16.605
Max.		14.5	18.9	35.3
Min.		3.9	4.81	4.4

No figures for hydroxyproline (3- or 4-) were obtained due to its absence or very low plasma concentration. It is not normally present in plasma. Its trial estimation to try and identify "the 440 proline peak" which occurred at the beginning of the analyser results, was negative. Also no spots occurred in the paper chromatograms which could have possibly been hydroxyproline, using isatin/Ehrlich Reagent detection technique (positive if purple red spot dull purple). This is a very sensitive technique, detecting as little as 0.1 µg/Sq. cm. of hydroxyproline if present.

Proline and arginine increase in plasma concentration to a moderate extent during perfusion, showing the usual sigmoidal curves. The fact that proline is only moderately raised indicates that any collagen breakdown taking place is comparatively small. The virtual absence of 3-hydroxyproline would support this. Ornithine virtually does not alter during perfusion, indeed all mean values are below the control mean value. This is interesting for whereas both proline and arginine are constituents of protein, ornithine is not. It is reasonable to assume therefore that the increase in proline and arginine during perfusion is due to some factor concerning protein metabolism which from the hepatic metabolism point of view could be due to one or both of the following :-

1) due to surgical procedures in cardiac bypasses the hapatic blood flow is not only altered but may be significantly reduced e.g. in the application of vessel clamps such as the aortic.

2) The degree of stress at an operation tends to reduce blood supply to tissues and organs except vital organs such as kidneys and brain.

These points will be raised again in the final discussion.

From the metabolic point of view these acids are closely connected. Ornithine serves in the formation of arginine from proline. It also is related to glutamic acid through glutamic acid semialdehyde. The latter is formed when hepatic transaminase acts on ornithine and a keto acid.

Proline on oxidation forms a pyrroline carboxylic acid which, adding the elements of water produces glutamic acid semi-aldehyde. The latter is thus the common factor of glutamic acid, proline and ornithine. The conversion of the latter to arginine is well known in the urea cycle.

Arginine participates in creatine synthesis with glycine, by forming guanido acetic acid (transamidination). Creatinine (creatine anhydride) was estimated in blood and

urine during and after some of the perfusions. Creatinine serves very much as an indicator of creatine destruction.

Arginine is increased in the blood and urine in muscular dystrophy but like histidine, arginine is not increased during A.C.TH or steroid therapy.

The hydroxylation of proline is an irreversible process, the product hydroxyproline follows a glucogenic route to form alanine and glucose.

The post operative urinary proline excretion. Fig. 10.29.

The urinary proline levels are greatly increased. It is of great interest that the maximal values obtained are due to the control operation. This is more than 100 mgms/24 hrs. above the maximal perfusion case. The other perfusion case "4 Ch." showed only a moderate proline increase in the first 4 post op. days then there was another marked rise between the 5th and 6th days to over 250 mgms/24 hours. This latter case thus shows considerable lag effect before maximal proline excretion occurs - suggesting possibly greater protein (collagen) breakdown in this post operative period.

Urinary ornithine Fig. 10.39.

This amino acid increased least during operation - a rise of only 2 or 3 mgms/24 hours being shown. This was in the control case only. Unfortunately standards were not

available when the perfusion cases were analysed and thus quantitation could not be done on these cases. From paper chromatography however it is clear that ornithine would come into the "least increase" category.

Urinary Arginine Fig. 10.42

Arginine showed very marked increases and there is virtually no difference between the perfusion cases and the control. Indeed "7 Mor" and the control are practically superimposed. These two cases show two peaks and "4 Ch." shows three peaks. These changes are of great interest in relation to Guillino's work on the protective effect of L-arginine.

HISTIDINE Fig. 10.23

	Control	1 Hr.	2 Hr.	3 Hr.
Mean	1.64	9.437	14.01	13.7
Max.		20.1	25.8	28.8
Min.		1.075	6.3	4.3

This essential amino acid showed moderate changes in perfusion, but after showing the characteristic rise in the first hour there is virtually very little further increase. From LD_{50} values of Guillino et al. it is one of the more toxic amino acids but as results show in these studies, the increases usually seen are not of significance.

Histidine is not increased in blood nor urine during ACTH therapy. This is interesting since it therefore cannot be used along with other amino acid data in the assessment of pituito-adreno-cortical secretion.

Histidine is increased in the urine during pregnancy. It is worth special mention that a large proportion of histidine is present in haemoglobin. Approximately 7.5 in human haemoglobin (using a M.W. of 64,500), see Tristram and Smith, 1963. From results of haemolysis it is surprising that the histidine concentrations have not risen more as free haemoglobin was present in the plasma in the order of 300-700 mgms/100 mls. Presumably this liberated haemoglobin in plasma did not undergo detectable or significant destruction during perfusion. This would explain why not only the histidine levels have not risen to a greater extent but also why the mean ratio of amino acid increases during perfusion bear no relation to the amino acid ratios in haemoglobin. If there had been any significant carnosine (β alanyl histidine) breakdown during perfusion (probably too early for such changes) then the plasma histidine concentrations would have been raised to a greater extent.

The catabolic phase of histidine metabolism proceeds to urocanic acid, by the action of an α deaminase. Urocanase

and water converts this acid to imidazolone propionic acid and on to formino-L-glutamic acid. Tetrahydrofolic acid (FH_4) then acts on the latter to produce "the 1 carbon carrier molecules" - N^5 formimino tetrahydrofolic acid and its N^{10} formyl FH_4 neighbour. These have been mentioned in the glycine metabolism.

1 and 3 methyl histidines are present in small concentration in plasma - mean 0.1 mgms/100 mls. They were just occasionally detected but no true increase occurred during perfusion. This would suggest that no significant breakdown of another dipeptide of muscle - anserine (β alanyl-1-methyl histidine) - was taking place during perfusion. It was of course of importance to look for 1-methyl histidine and histidine in post operative urines - and this was done.

Post operative urinary histidine excretion. Fig. 10.41.

These results show marked differences. A large increase occurs in "4 Ch." but a relatively small increase in the other perfusion case "7 Mor".

The control values take a "middling" course and only reaches a maximum on the 5th day (no further urine samples were able to be obtained). It is reasonable to assume that a decrease in concentration would occur soon after this.

It has already been mentioned above that histidine is one of the most toxic amino acids and it is interesting that these three operations show such relatively diverse changes in their excretory pattern.

The 1 and 3 methyl histidines were present in moderate concentration in the urines as judged by both analyser and paper chromatography results. They could not be quantitated however due to absence of standards.

METHIONINE Fig. 10.20.

	Control	1 Hr.	2 Hr.	3 Hr.
Mean	0.59	0.94	1.53	2.79
Max.		1.9	3.67	4.2
Min.		0.3	0.48	0.76

This essential amino acid normally present in small concentration in the plasma showed a similar sigmoidal increase like other amino acids but on a diminutive scale.

Methionine plays a significant part in metabolism in donating it's methyl group (through S-adenosylmethionine) in the synthesis of choline and creatine. The methyl group of anserine is also derived from methionine. Transamination with α ketoglutaric acid in the liver yields α keto γ methylthiobutyric acid. The sulphur of methionine is used in the synthesis of cysteine and cystine (see below).

Demethylation and hydroxylation of methionine results in serine which in the presence of the liver enzyme cystathionine synthetase and pyridoxal phosphate forms cystathionine. This amino acid is present in only small quantities in plasma and was virtually undetected per se in plasma or urine. This is probably because an hepatic cleavage enzyme - thionase - in a hydrolysis reaction splits the cystathionine molecule in two, producing cysteine and homoserine. The latter eventually forming propionic acid and glucose.

Post operative urinary methionine excretion. Fig. 10.34

Interesting results were obtained. Both the perfusion cases showed virtually no change in concentration, whereas the control showed a great increase from 40 to 313 mgms/ 24 hours. The urinary results of the perfusion thus compliment the plasma findings. If active methionine metabolism is taking place then it is clear that in order for the plasma methionine concentration to remain constant, active methyl donation, transamination, demethylation and hydroxylation must be taking place - thus giving rise to the associated products of choline, creatine, answerine, cystathionine, serine, cysteine and homoserine etc.

It is difficult to explain why such a profound increase was found in the control operation.

CYSTEINE (and Cystine) Fig. 10.24.

	Control	1 Hr.	2 Hr.	3 Hr.
Mean	1.14	3.34	6.98	11.5
Max.		5.82	9.33	13.5
Min.		0.137	0.12	9.73

These non essential acids were measured as cysteine values. The changes seen during perfusion show a sigmoidal type increase but the change is more acute over the 2-3 hour period. As mentioned above cysteine is derived from methionine through cystathionine, and cystine via cysteine. Most of the urinary sulphur is derived from the sulphur amino acids and is found either unchanged as the latter, or as sulphates (after oxidation), the latter are both inorganic and ethereal.

Homocysteine - the demethylated relation of methionine was not assayed in the plasma (nor urine) samples. It was not present in significant concentration in either.

It is significant to mention that the sulphur containing amino acids and related compounds do appear to be in a "state of flux" during perfusion and the post operative period as seen by the cysteine results. The low methionine values certainly need not suggest an inactive metabolic state since any potential increase in this amino acid could be easily nullified by its participation in many sulphur metabolic pathways, e.g. creatine, choline,

cysteine and sulphate formation as well as its own end products - glucose.

Post operative urinary cysteine excretion. Fig. 10.33.

Both perfusion cases showed very similar results of large increases in the post operative period. The maximal excretion lasted over the first, second and third days. Unfortunately cysteine values for the control were not able to be calculated due to absence of standard.

These results are most interesting for it could suggest - as mentioned in the methionine discussion that this latter amino acid is contributing to cysteine formation and its excretion. The plasma cysteine concentrations have been seen to rise during perfusions but not to any great extent - however the ready excretion in the urine could be explained by

1) A low cysteine renal threshold.
2) Active excretion and limiting (limited) tubular reabsorption.

Cystinuria is one of Garrods inborn errors of metabolism. Cysteine is concerned in the formation of mercapturic acid. This is an important detoxication pathway. Cysteine is acetylated (α amino group) before conjugating with

products to be detoxicated such as benzene and halogenated aromatic hydrocarbons. One of the few examples of this mechanism is the detoxication of bromobenzene which is excreted as p-bromophenylmercapturic acid.

It can therefore be hypothesised that if

1) liver metabolism is retarded (due to a reduction in blood supply during perfusion) and
2) excessive urinary loss of cysteine occurs post-operatively :

then it is quite possible that a reduction in this detoxication mechanism may occur, and thus give rise to a temporary increase in circulating toxic products. It is also quite possible that other detoxication mechanisms may also suffer due to similar causes.

Cysteine and cystine are increased in March haemoglobinuria. In these perfusion cases a significant increase in haemolysis has been demonstrated. This free haemoglobin is excreted in the urine when the plasma concentration is in excess of 150 mgms/100 mls. Thus the very pronounced increase in urinary cysteine could be largely accounted for by this significant haemoglobinaemia.

TRYPTOPHAN

This essential amino acid which is normally present in small quantities in plasma (approx. 1.0 mgms/ 100 mls.) showed no increase during perfusion and was only barely detectable. In its absence negative nitrogen balance and tissue wasting rapidly ensue, together with a fall of serum albumin and globulin and haemoglobin.

Tryptophan is one of the most toxic amino acids (Guillino et al) but its importance is negligible in perfusions due to the above facts.

Tryptophan occurred in negligible concentration in the urine.

TAURINE.

Taurine appears in low concentrations in the plasma but high concentrations in the urine. As standards were generally not available this amine had to be omitted from the quantitative analysis. However it regularly appeared in the urine as judged by paper chromatographic results.

It is of interest that taurine is greatly increased, like certain other amino acids in muscular dystrophy and in pernicious anaemia.

Taurine is formed in liver from cysteine sulfinic acid either by oxidation and subsequent decarboxylation or vice versa of these two processes. Two other amino acids deserve special mention and these are :

β alanine and β -amino isobutyric acid. B.A.I.B.

β alanine appeared as a significant peak on the automatic analyser results between phenylalanine and the pre ammonia peak (ornithine). It also was well defined in various paper chromatograms a selection of which is shown. It was also constantly present in pre-operative specimens.

β amino isobutyric acid is normally absent from the urine. Its presence however is genetically determined. It does appear in pathological conditions such as leukaemia, starvation and March haemoglobinuria. Gartler, 1959 suggests that Thymine is the only source of this amino acid in man and its increase during nucleoprotein catabolism (leukaemia, neoplasms etc.) thus explained. Thymine undergoes reduction to its dihydro form and cleavage of the ring system produces β -ureido-isobutyric acid from which B.A.I.B. is formed.

B.A.I.B. excretion is little influenced by ordinary dietary variations. It is increased however in the urine under fasting conditions of 2-4 days.

B.A.I.B. appeared to increase in moderate concentrations in the urine during post perfusion and post-op, control periods. This could be due to several factors.

1) pre-operative starvation (probably minimal effect)

2) protein catabolism

3) Decrease or absence of enzyme responsible for degradation of B.A.I.B. to methyl malonate semi aldehyde (assuming no genetic defect).

Both β alanine and B.A.I.B. were not quantitated due to standard not being available. In the table 10.1 of mean control plasma and urinary amino acids values these two acids are classed together since β alanine is β amino propionic acid and B.A.I.B. is ∝ methyl β amino propionic acid.

Summary of plasma amino acid changes in perfusion and non perfusion operations

The results show that during perfusion the amino acids may be roughly divided into three arbitrary groups.

Severe changes: Aspartic and glutamic acids, leucine and lysine.

Moderate changes: Threonine, serine, proline, glycine, alanine, valine tyrosine, phenylalanine, histidine, arginine and "ammonia".

Slight changes: Cysteine, isoleucine, methionine, hydroxylysine, ornithine and tryptophan.

The control operation "15 McC" of 3 hours duration shows an increase in all amino acids, the values of which

are roughly similar to the changes seen in the 30-45 minute perfusions. As in the perfusion cases the largest increase of amino acids seen in the control operation was with glutamic acid, leucine and lysine.

The moderate and slight changes of the control were similar to the perfusion results above.

Summary of urinary amino acid changes in the post operative period

All the urinary amino acids increased in the post operative period with the exception of methionine (perfusion cases only). There was a similar 4-7 fold increase of amino acids in the control operation "15 McC".

The following amino acids showed virtually no significant difference between the perfusion results and the control operation :-

Aspartic acid	x 5	Alanine	x 6-7
Threonine	x 4-5	Lysine	x 8
Serine	x 5	Hist.	x 5-6
Glutamic acid	x 8	Arg.	x 6
Glycine	x 6-8		

All were very much increased as indicated.

Valine, isoleucine, leucine, tyrosine and phenylalanine were all greatly increased, (6-8 fold), in the perfusion cases but the control operation values were low in comparison.

The control values of proline and methionine were greater than the perfusion values. With proline this difference was not great, but with methionine there was great dissimilarity between the perfusion results and control results, (5 fold increase). The methionine perfusion values increased very little.

Ornithine and hydroxylysine (control operation only) also showed only a diminutive increase in the post operative period. Results of these amino acids in post perfusion patients could not be obtained.

After acid hydrolysis - as was expected - there was a large increase in aspartic and glutamic acids and glycine from conjugates. The aspartic and glutamic acid increases are virtually all due to liberation from these conjugates since they are both present in only small quantities in the free state. 50% approximately of the liberated glutamic acid is due to phenylacetylglutamine and approximately 70% of the liberated glycine is derived from hippuric acid.

It is of interest that the control operation mimics the post perfusion results very closely in regard to these three amino acids.

Although acid hydrolysis liberates other amino acids - hitherto bound - these are of much smaller concentration.

It is difficult to equate the findings of plasma amino acid concentrations with the results found in the urines because :-

1) The participation of each amino acid in metabolism is an autonomous unit, playing large or small significance. Alanine which appears in appreciable concentration in plasma and urine is a non essential acid with comparatively little responsibility in metabolism. On the other hand an essential amino acid like methionine which is present in low concentrations in plasma and urine (perfusion cases only) plays a vastly important role in sulphur and general metabolism.

2) It has been pointed out in the introduction of this Section (10) that when certain amino acids (if not all) are increased substantially in the plasma they block and/or alter the excretion and reabosprtion of other amino acids, c.f. hereditary prolinuria.

3) Associated with (2) are the specific renal threshold of individual amino acids which in turn are partially dependent on specific enzymic activity in the renal tubules.

4) Concerning hepatic metabolism during surgery and post operatively - any deficiency in hepatic enzymic activity concerned in the degradation of amino acids, the formation of new ones (e.g. cysteine from methionine and arginine from proline), the transamination/deamination reactions, or the responsibility of the detoxication mechanisms - will lead to a differential increase in plasma amino acid levels, as the rate of excretion of each amino acid from the renal tubules is dependent on 2 and 3 above (and possibly other unknown factors), - the urinary excretion of each acid bears very little resemblance to the plasma findings. This would appear to be a rational explanation for the amino acid results from these perfusion cases.

Hyperaminoacidaemia is well known to occur in liver disease - and increase proportionately with the severity. Ankeney et al. 1961 has shown that temporary hepatic dysfunction does occur during perfusion and involves tyrosine and methionine mainly and to a lesser extent phenylalanine and threonine.

It has been shown by Newburg - as long ago as 1925 that intravenous injections of amino acids leads to injurious effect on the renal system. This has been more recently substantiated by Guillino. The most toxic amino acids from the renal toxicity point of view are

tryptophan
tyrosine
histidine
and lysine

Tryptophan was found to be barely present in plasma during perfusion and in the urine post operatively.

Tyrosine only increased very moderately during perfusion but reached appreciable urine concentrations post operatively.

Histidine only increased moderately during perfusion but again reached high urinary concentrations post operatively.

Lysine increased significantly during perfusion and also in the post operative urines.

It must be emphasised that an amino acid of one concentration has not necessarily got the same degree of toxicity as another amino acid present in the same concentration.

The protective effect of L-arginine on the $L.D._{50}$ values of rats fed with varying concentrations of 9 other amino acids is of great interest because - as can be seen in the graphs of this section - this amino acid also increases during perfusion (moderate increase) and in the urinary post operative specimens. Thus it is plausible to hypothesise that this may have a protective function in its own right. In higher concentrations however arginine is also known to be toxic.

Why L-arginine appears to have a protective effect against mortality may be due to increased mobilisation of the urea cycle. Liberation of amino groups or free ammonia from amino acids may be achieved by amino acid oxidases or transamination reactions. Ammonia and other toxic substances may be produced at a rate in excess of the capacity of the body to utilise them. L-arginine may well mobilise the urea cycle to utilise these excess nitrogen metabolities. Krebs showed in 1932 that urea formation from ammonia by liver slices was greatly increased upon the addition of arginine or ornithine.

Apropos of the latter - the ornithine values in plasma during perfusion and in the urines post operatively was in low concentrations and showed no increase in either body fluid. So it is reasonable to assume that any beneficial effect ornithine has is virtually negligible bearing in mind these low concentrations.

FIG. 10·2 "2.P.M." Plasma Amino Acids : Control and Final

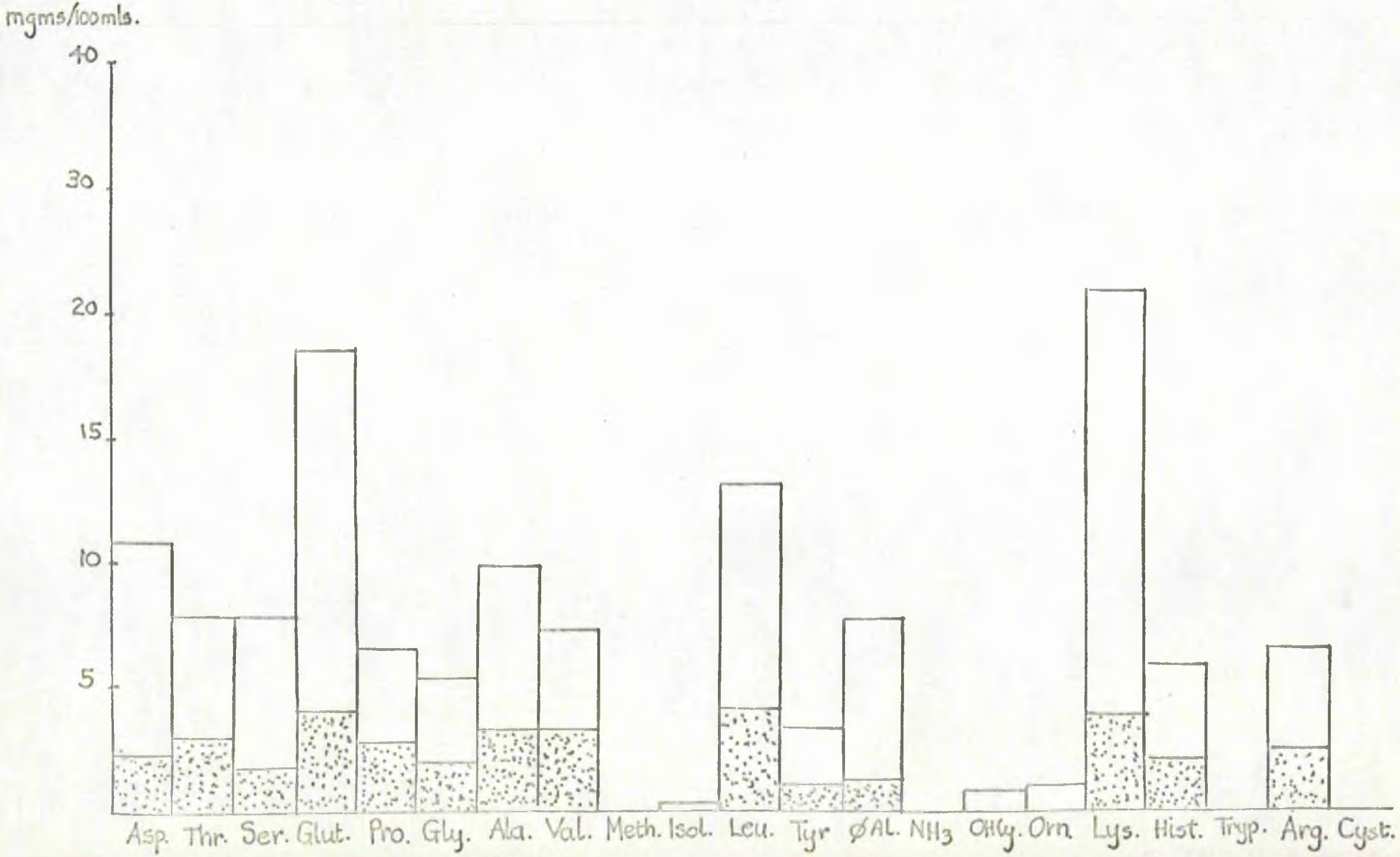


FIG. 10·3 "3.B.K" Plasma Amino Acids : Control and Final.

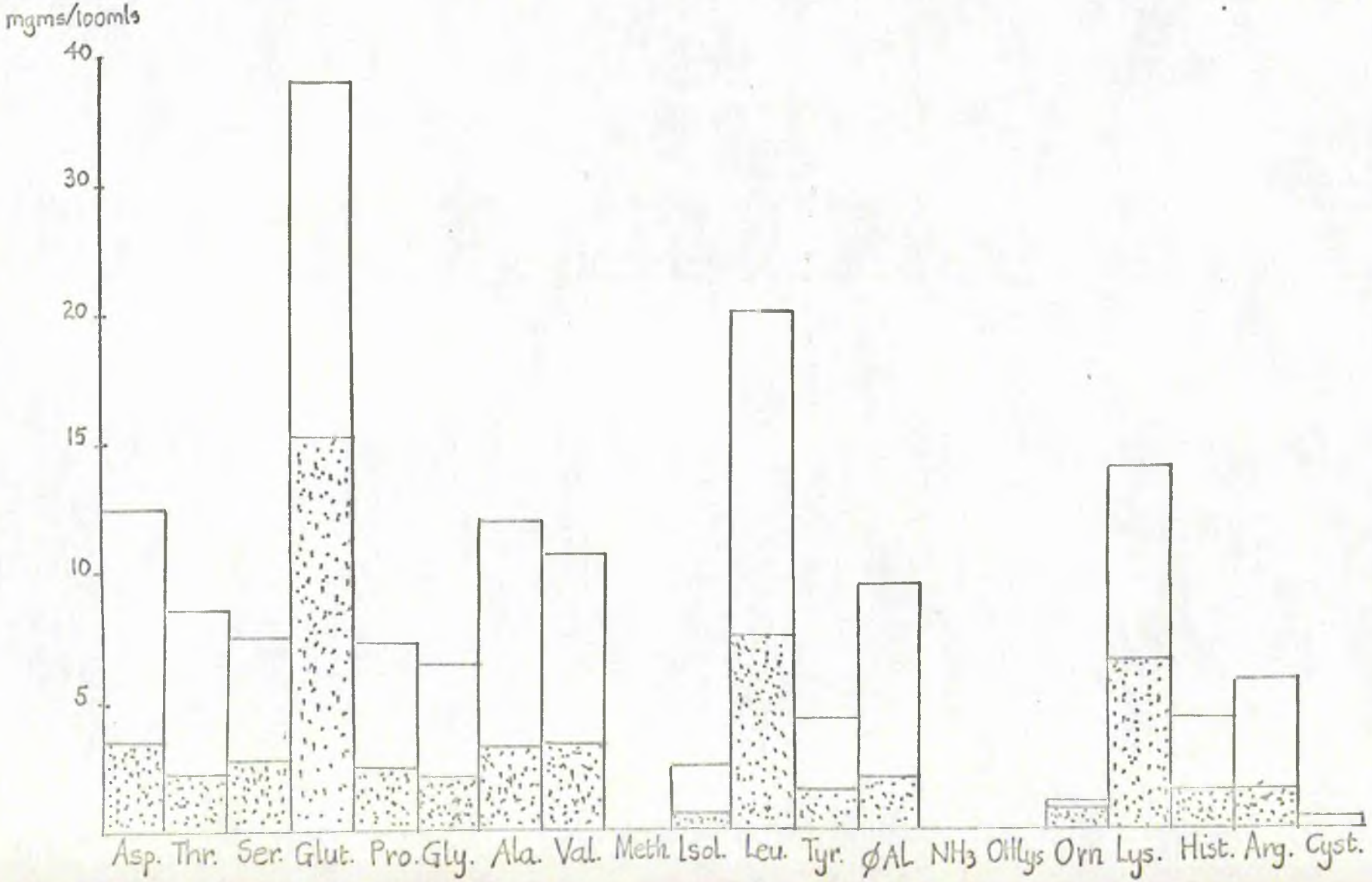


FIG. 10.4 "4 M.Ch." Plasma Amino Acids: Control and Final.

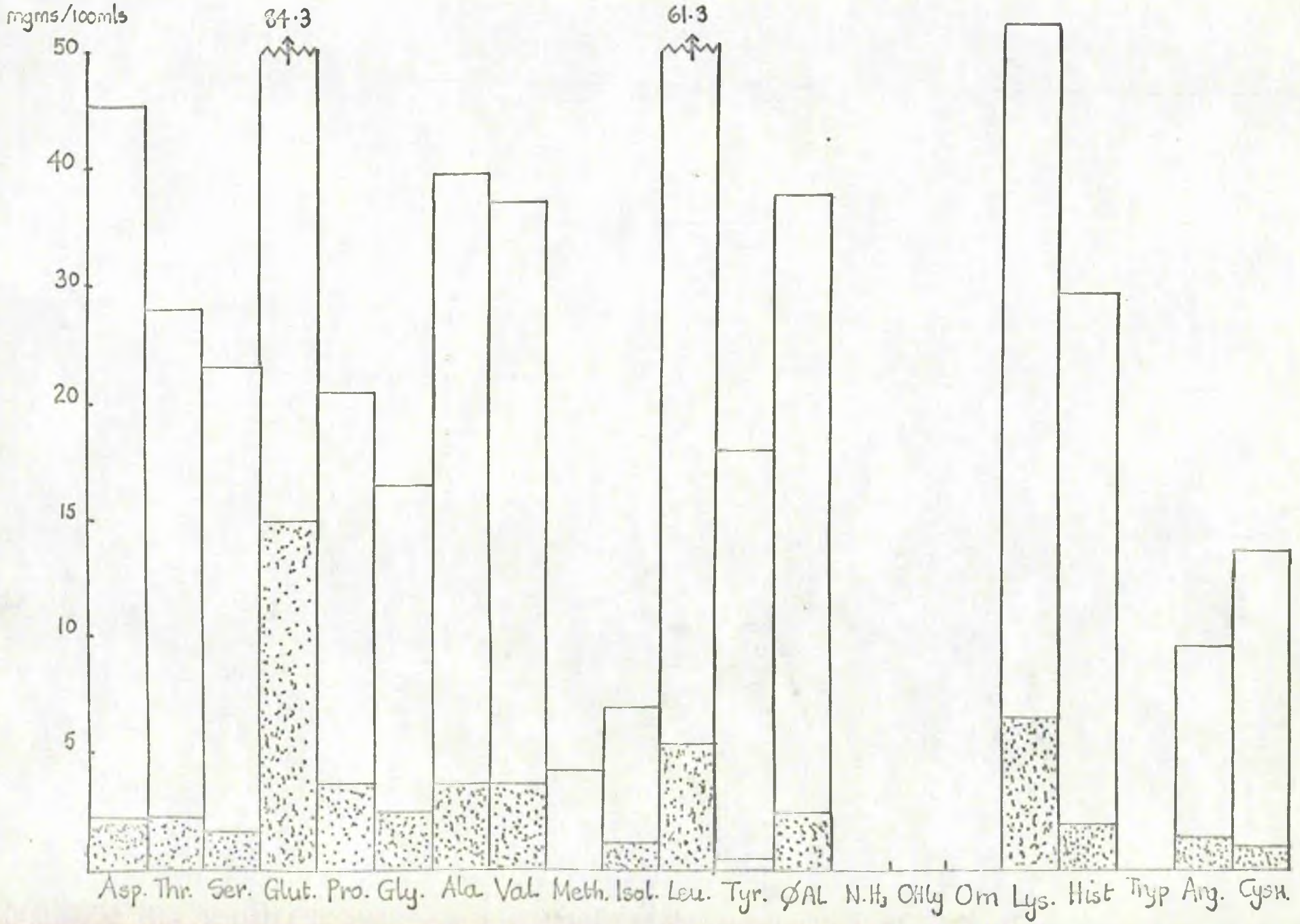


FIG 10·5 "5.I.D." Plasma Amino Acids : Control and Final.

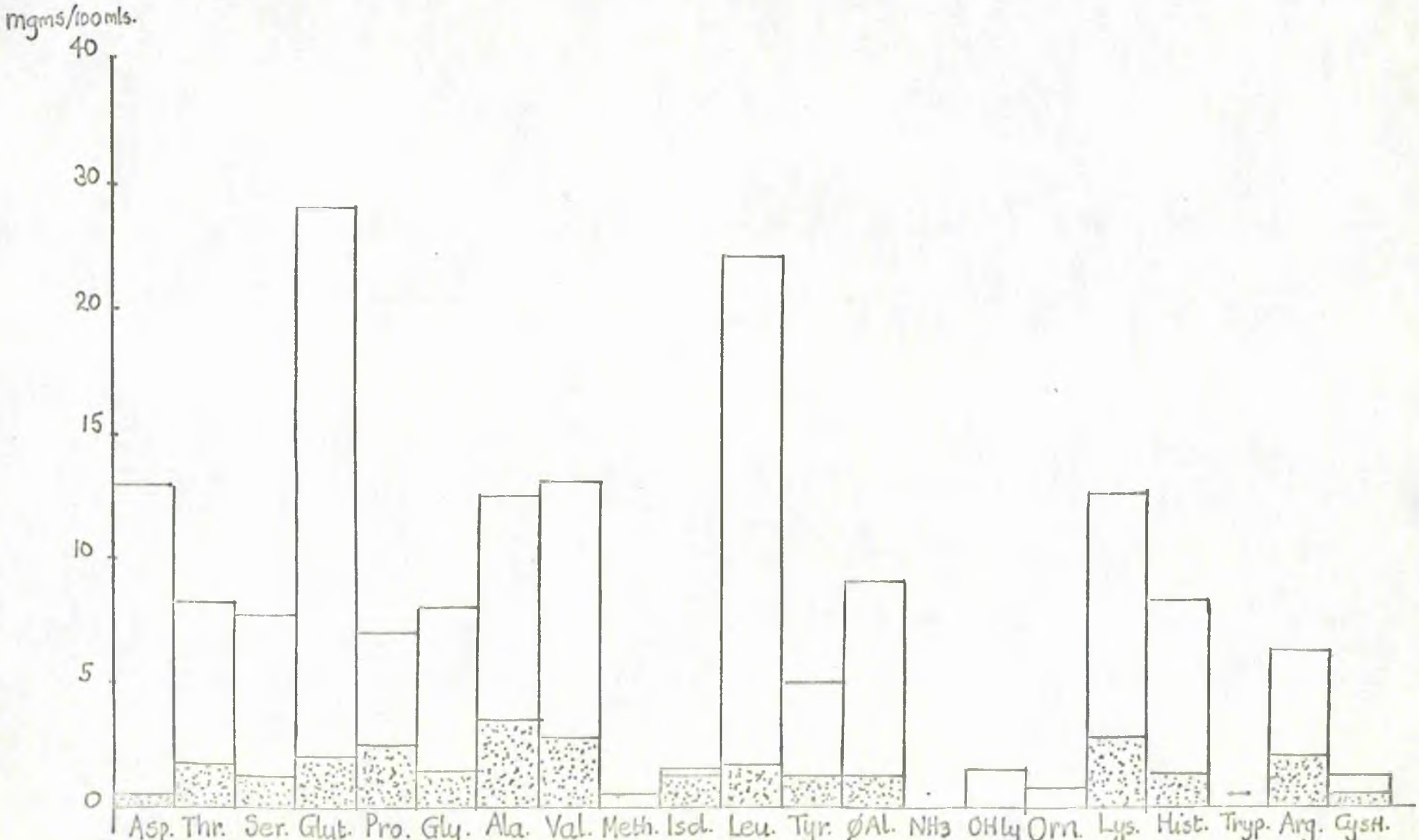


FIG. 10·6 "6.5.5" Plasma Amino Acids: Control and Final.

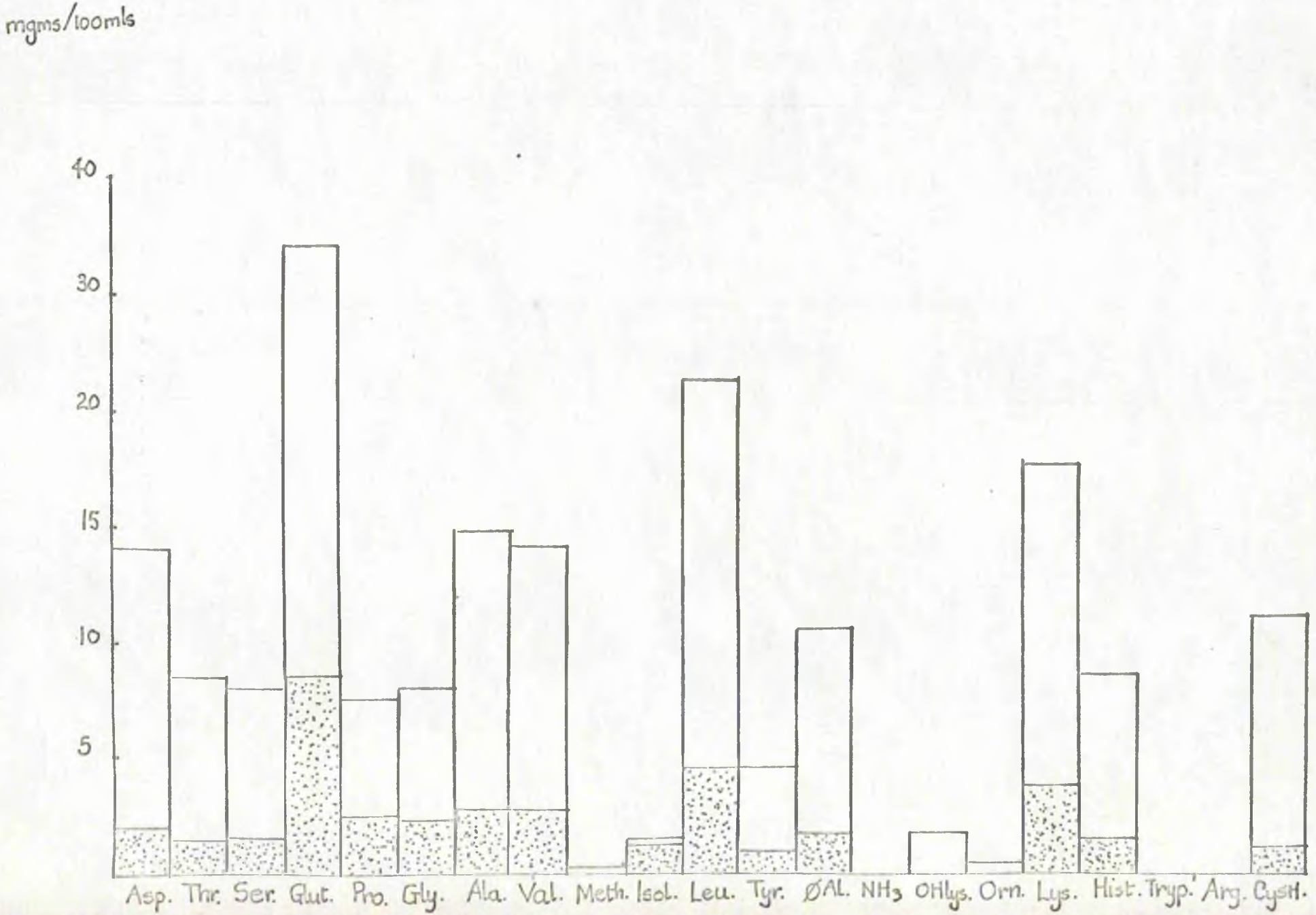


FIG. 10·7 "7 D.M" Plasma Amino Acids: Control and Final.

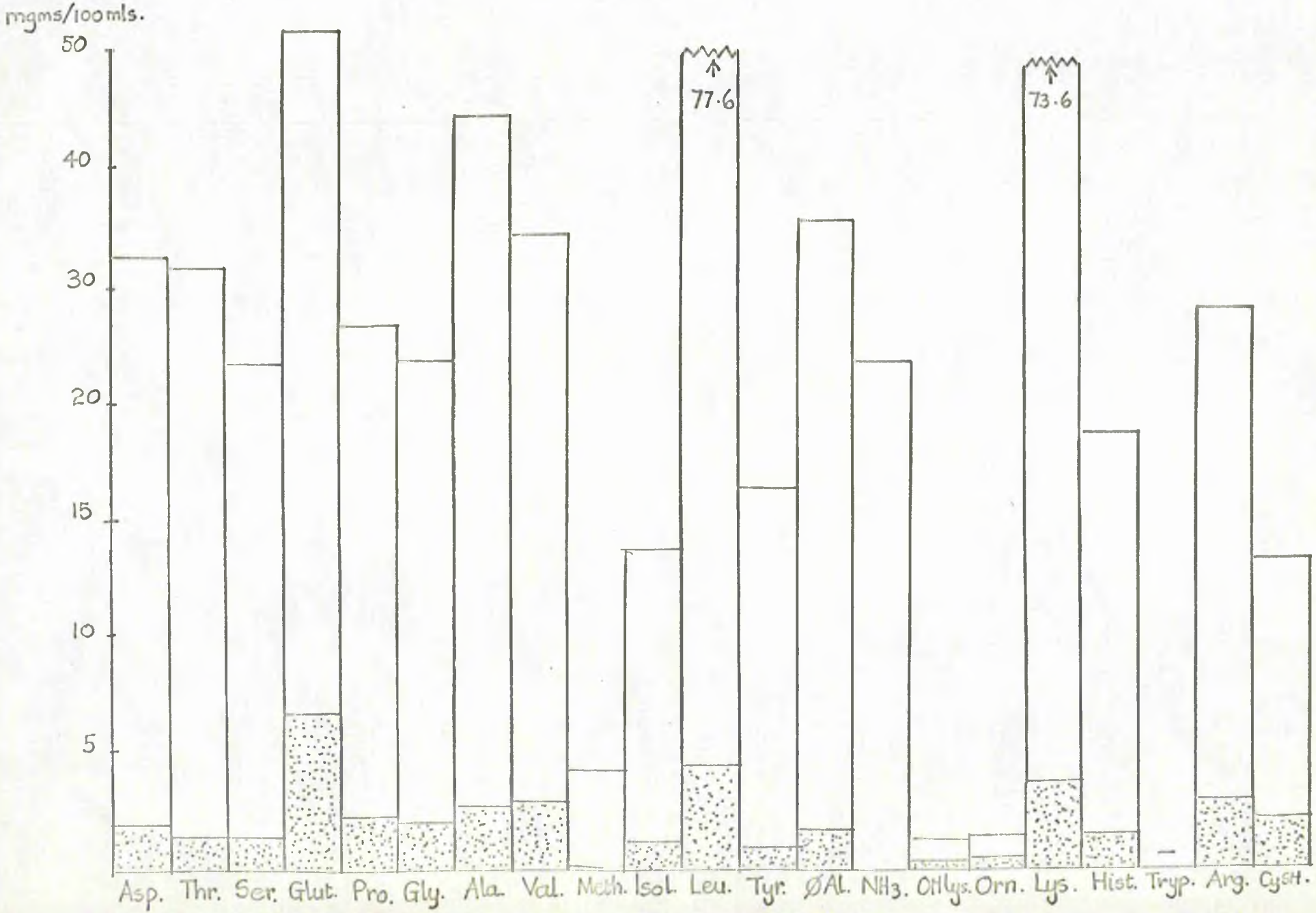


FIG. 10·8 "S.F.K" Plasma Amino Acids: Control and Final.

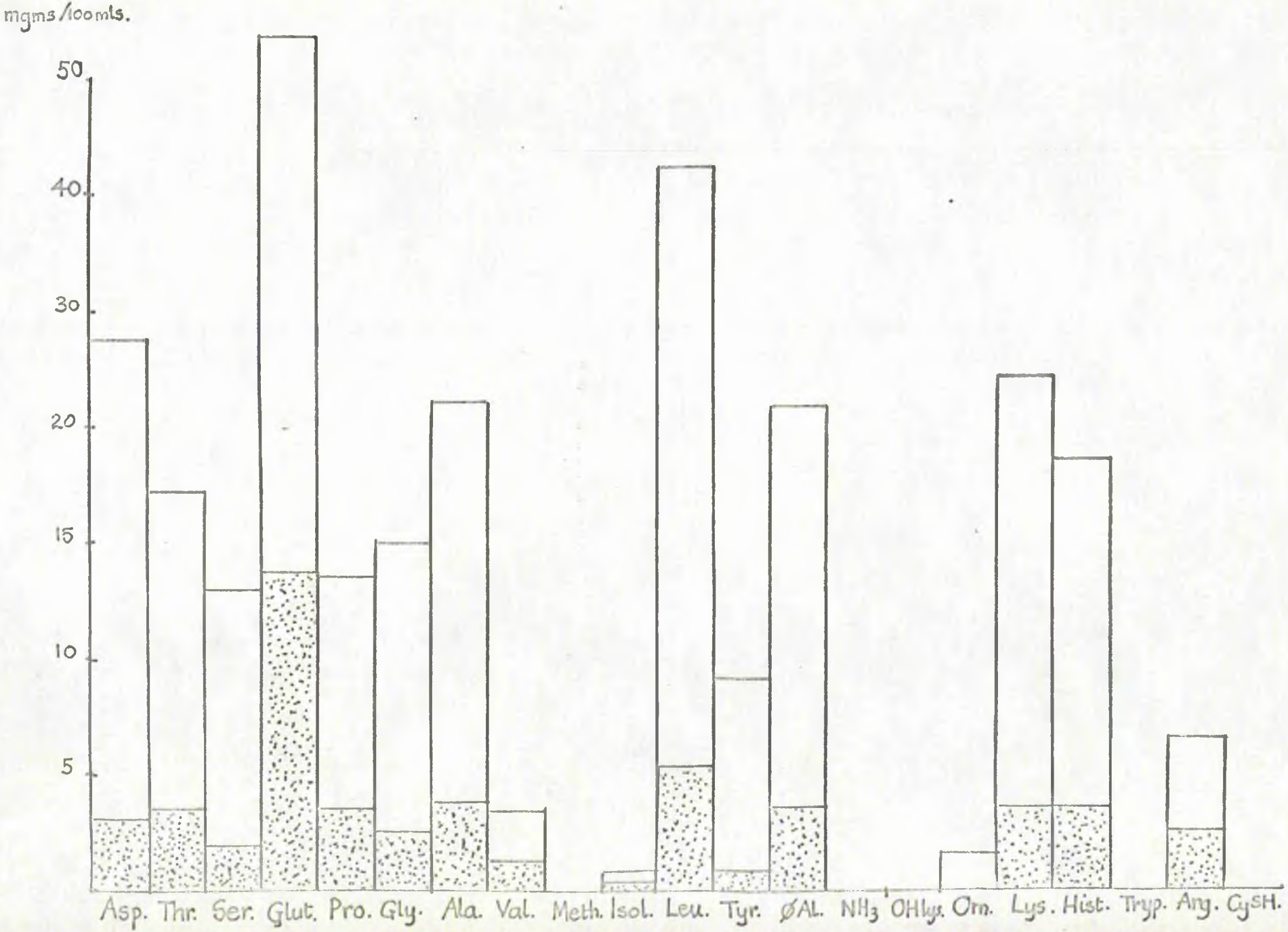


FIG. 10·9 "9.C.N" Plasma Amino Acids: Control and Final.

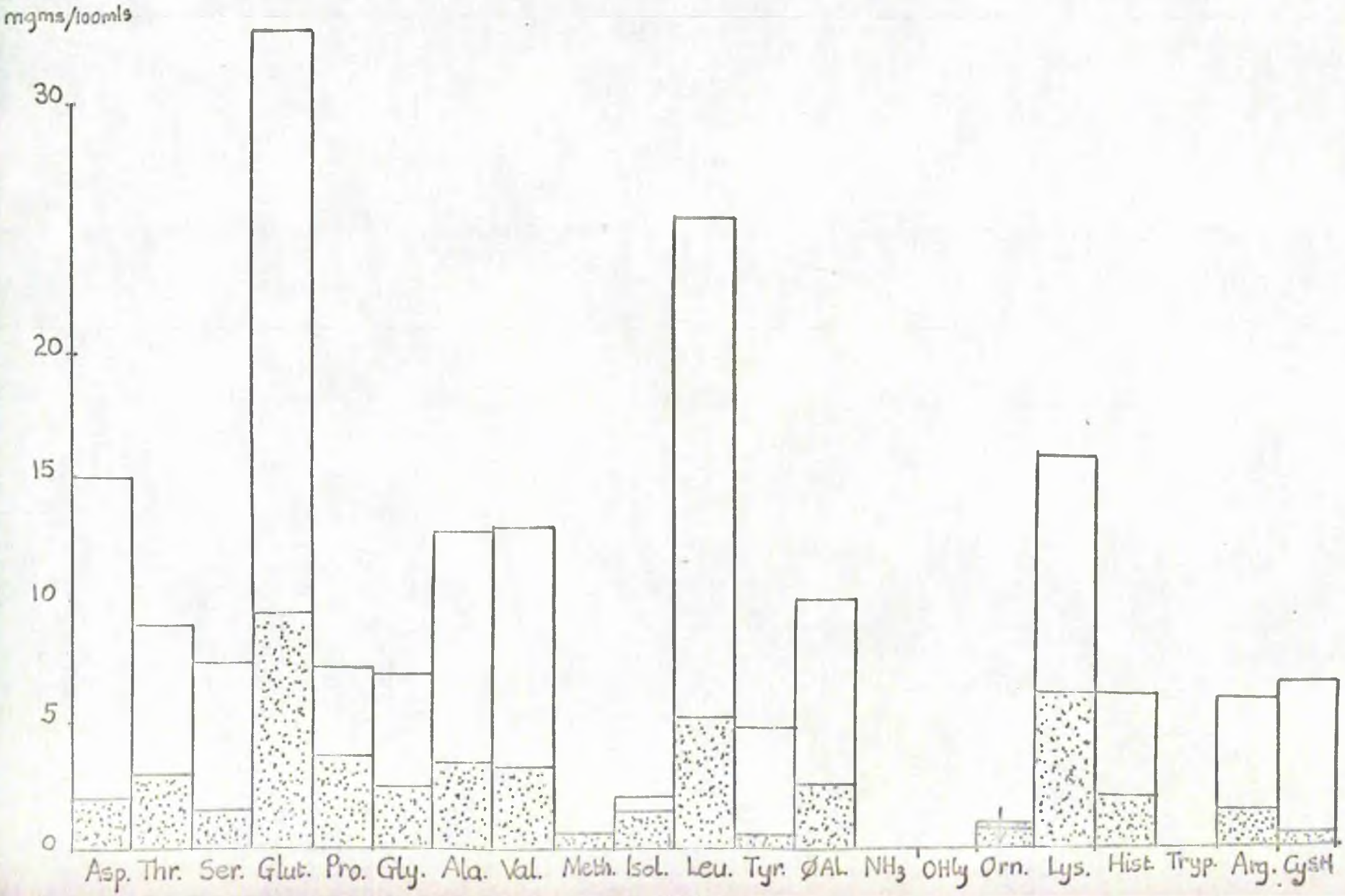


FIG. 10·10. "10.R.M." Plasma Amino Acids: Control and Final.

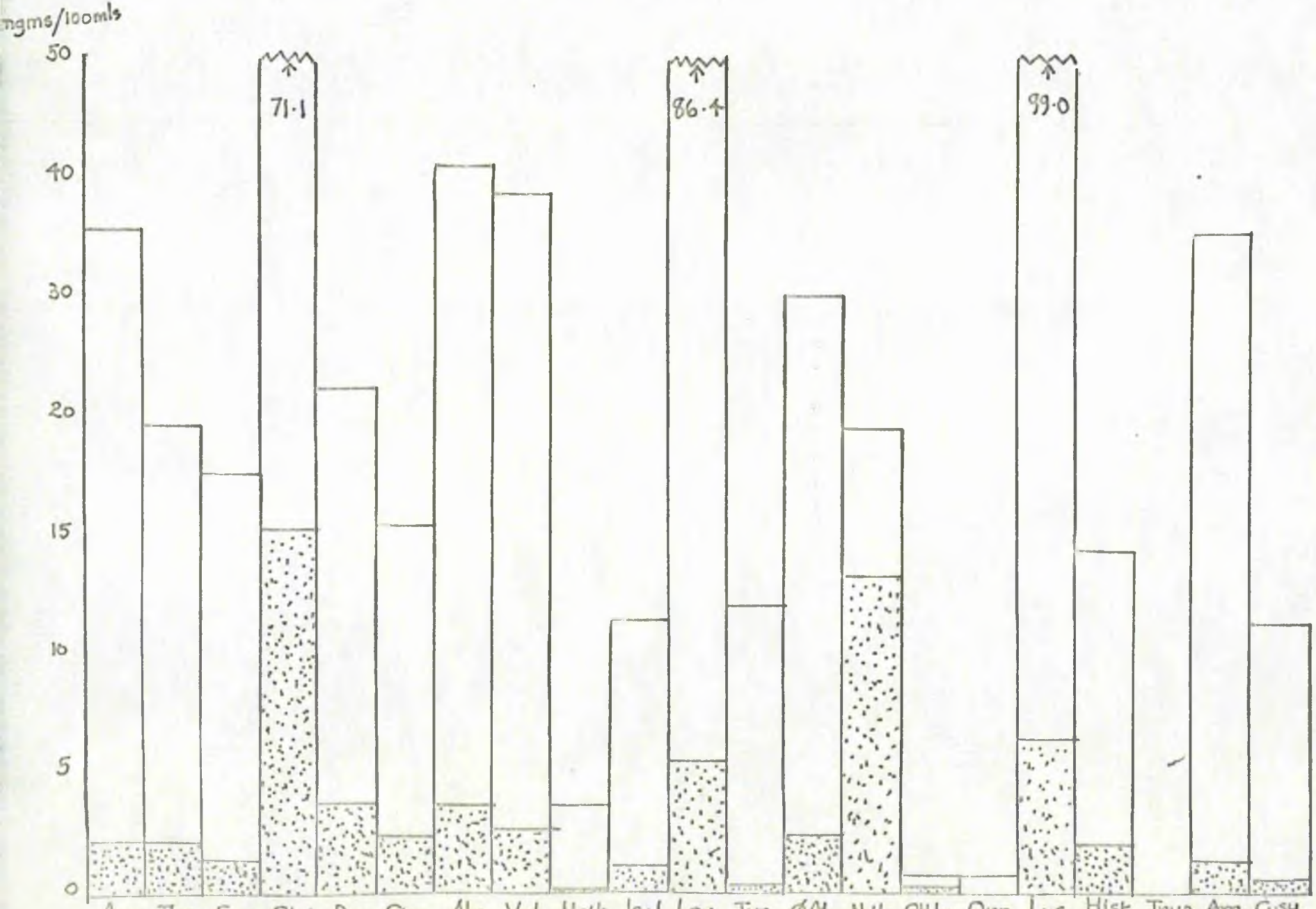


FIG. 10·11. "11.A.C." Plasma Amino Acids: Control and Final.

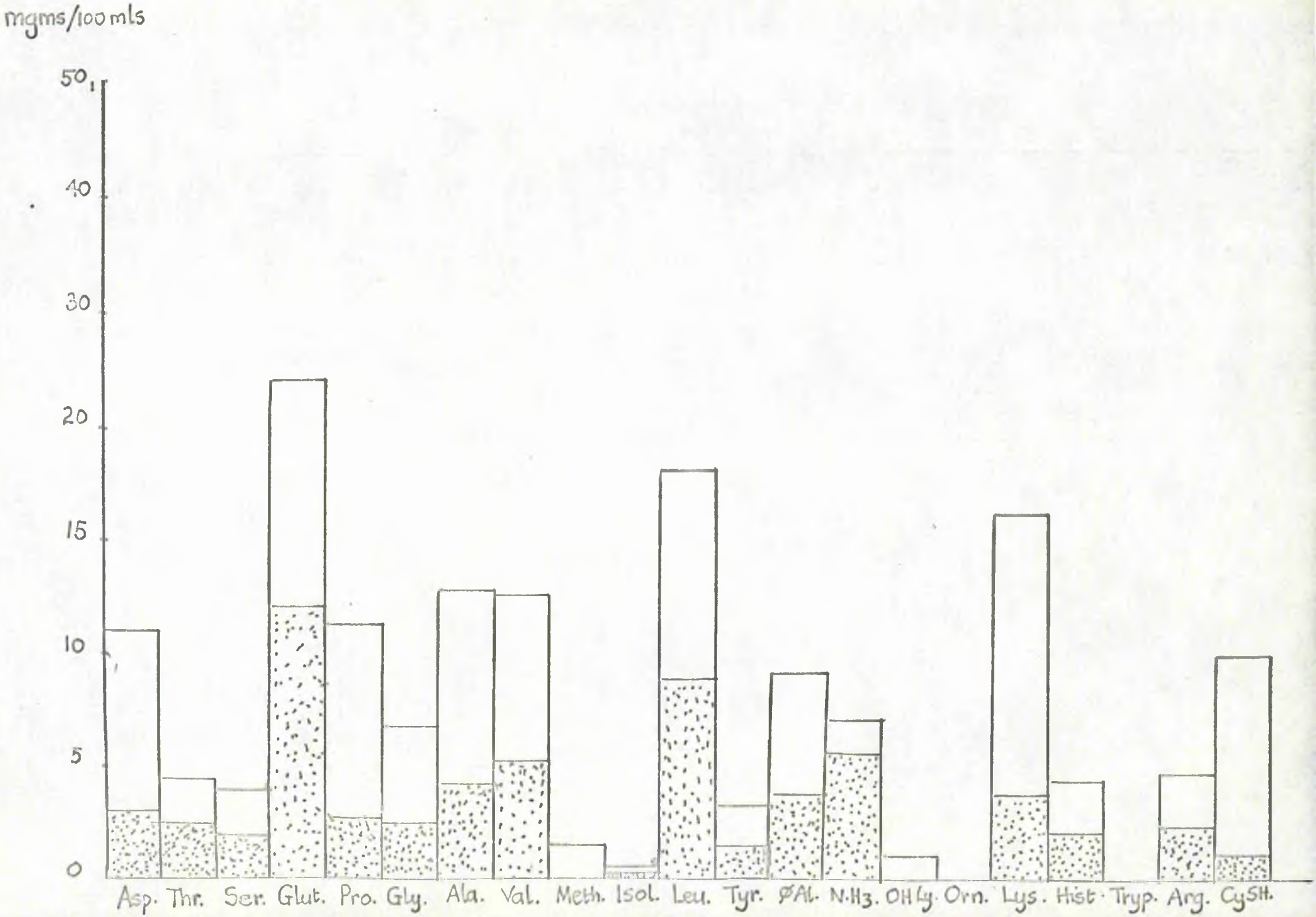


FIG. 1012. "12. D.S." Plasma Amino Acids: Control and Final.

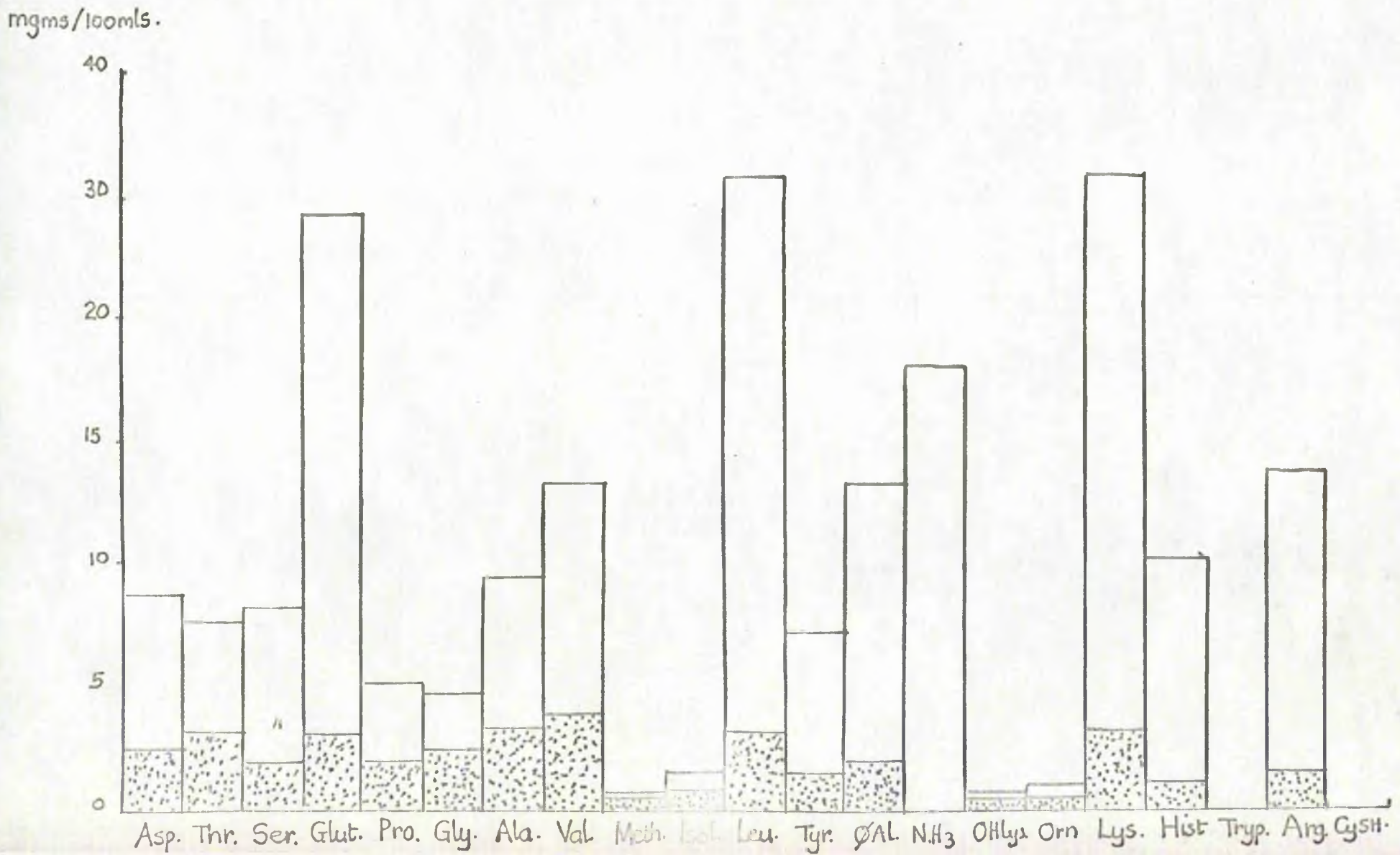


FIG. 10:13. "13. R.D" Plasma Amino Acids: Control and Final.

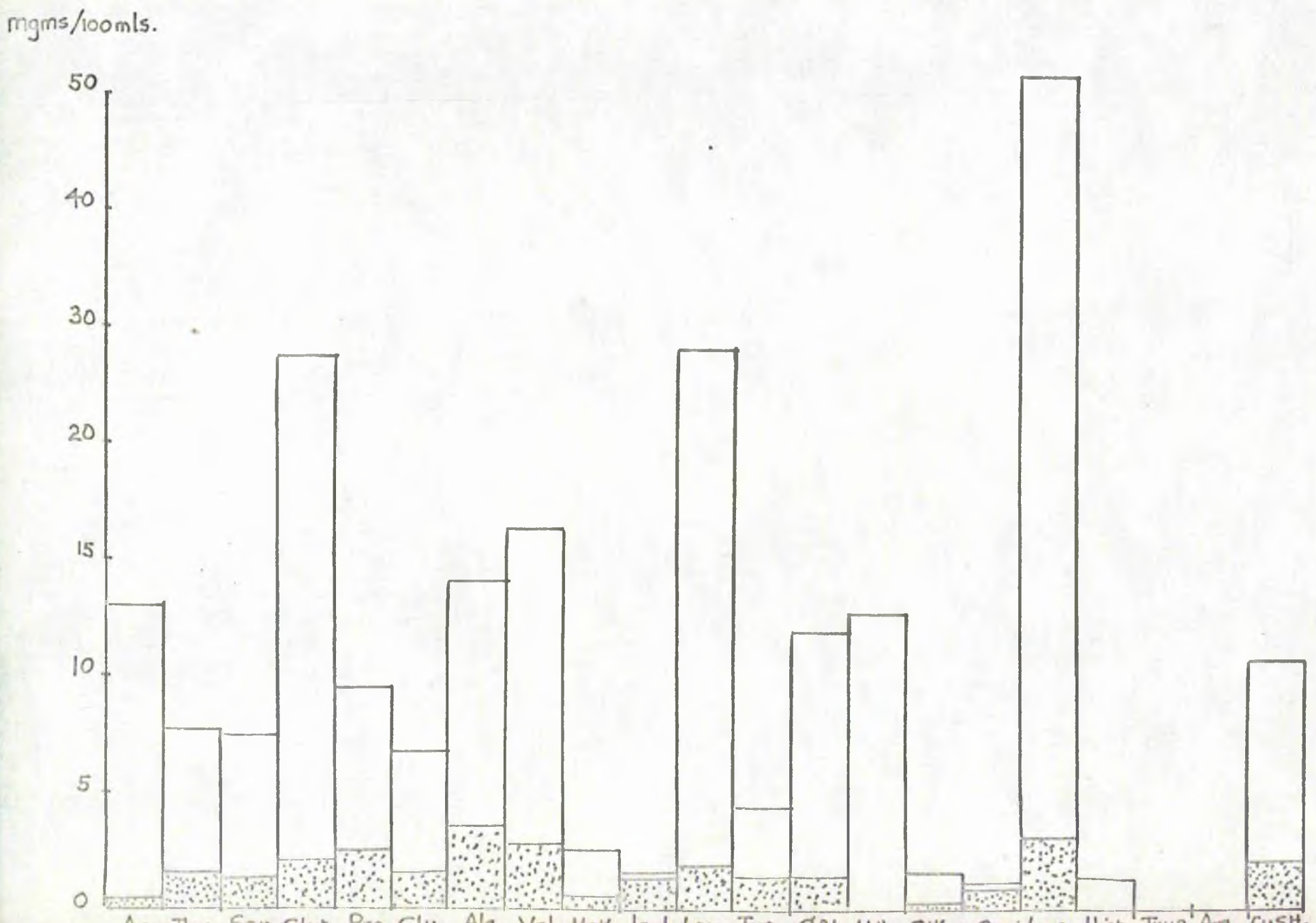


FIG. 10·14. "14: H.D." Plasma Amino Acids: Control and Final.

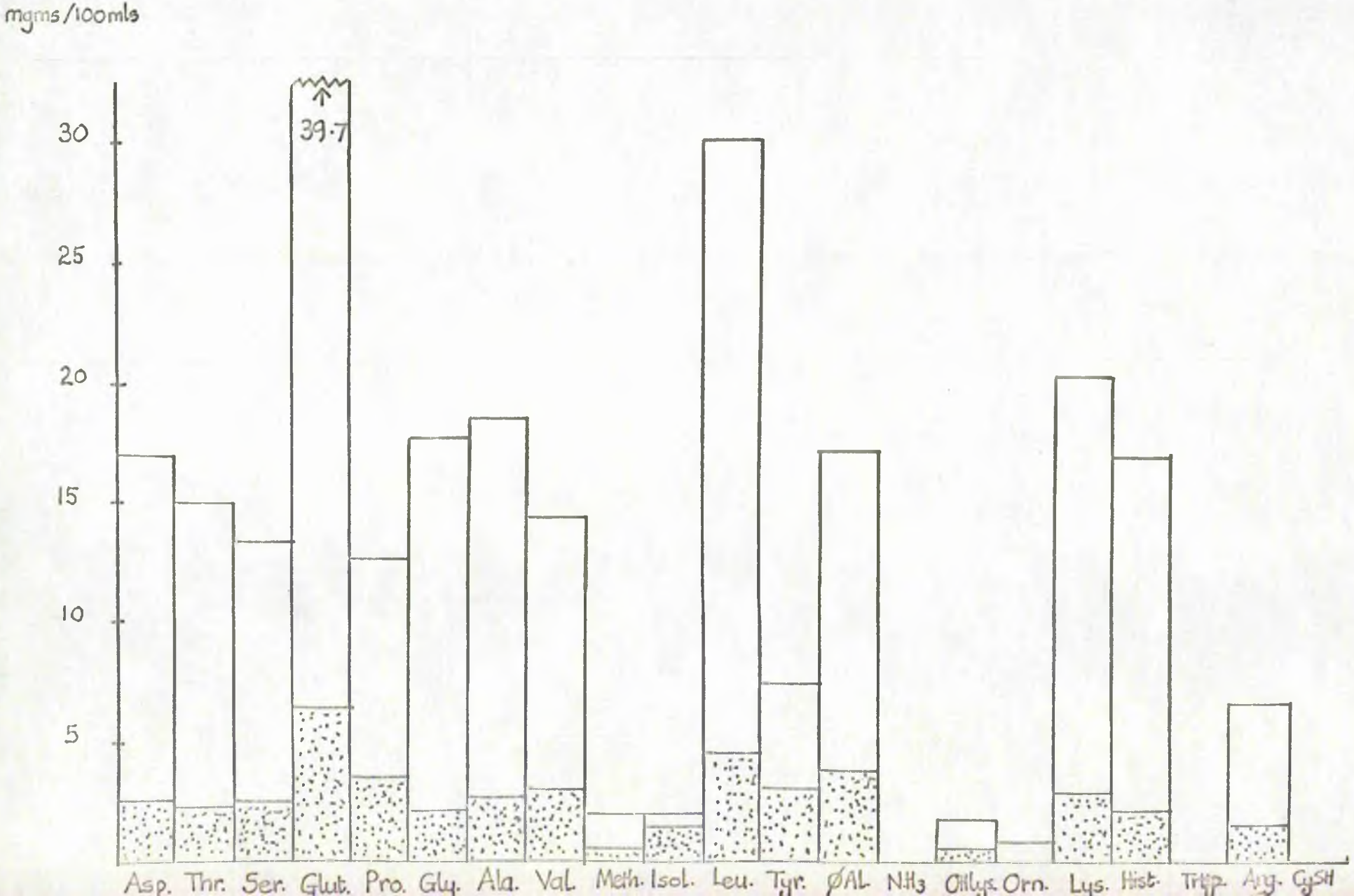


FIG. 10-15. "15. J. McC." Plasma Amino Acids : Control and Final.

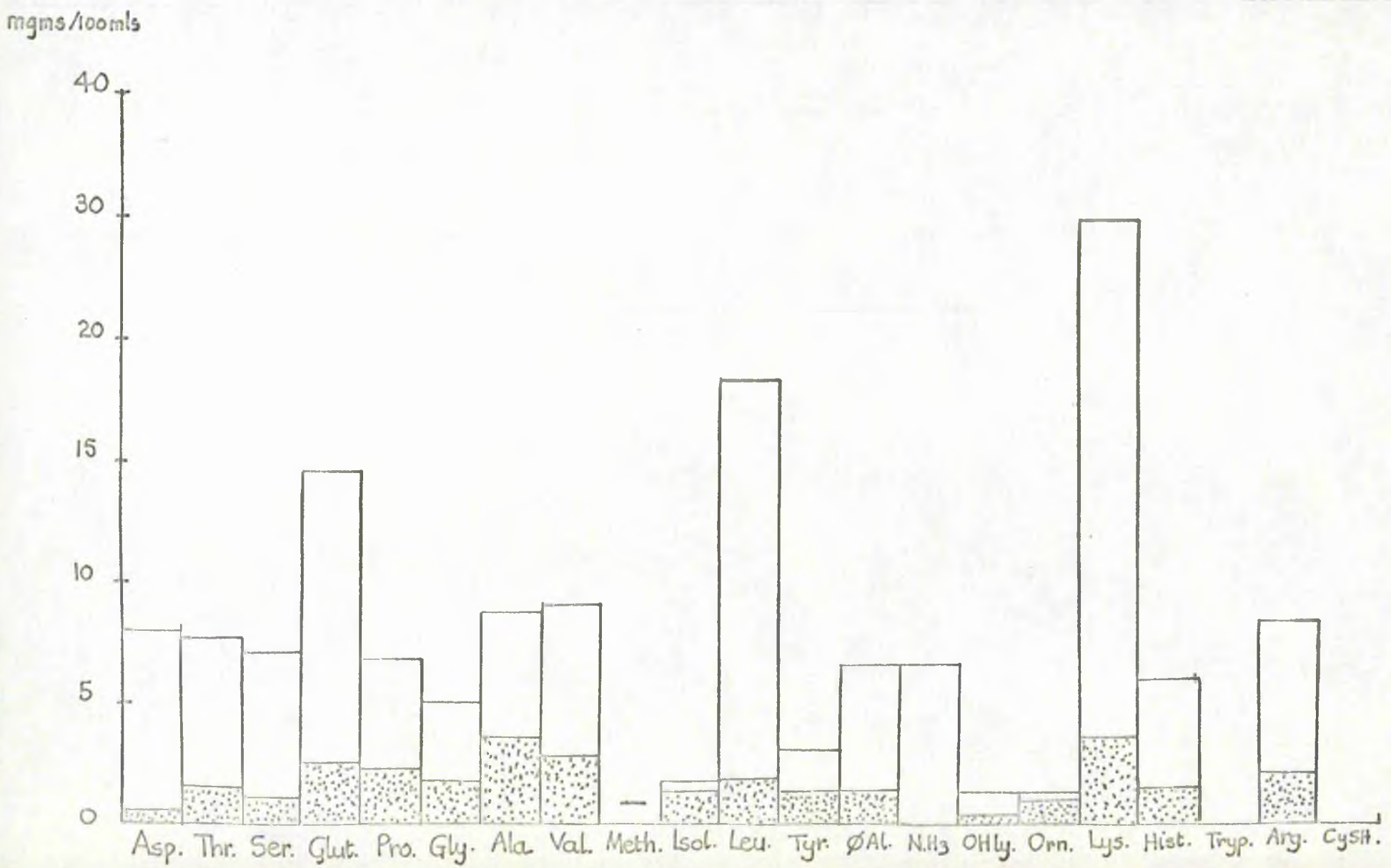


FIG. 10.16. "16.K.R." Plasma Amino Acids: Control and Final.

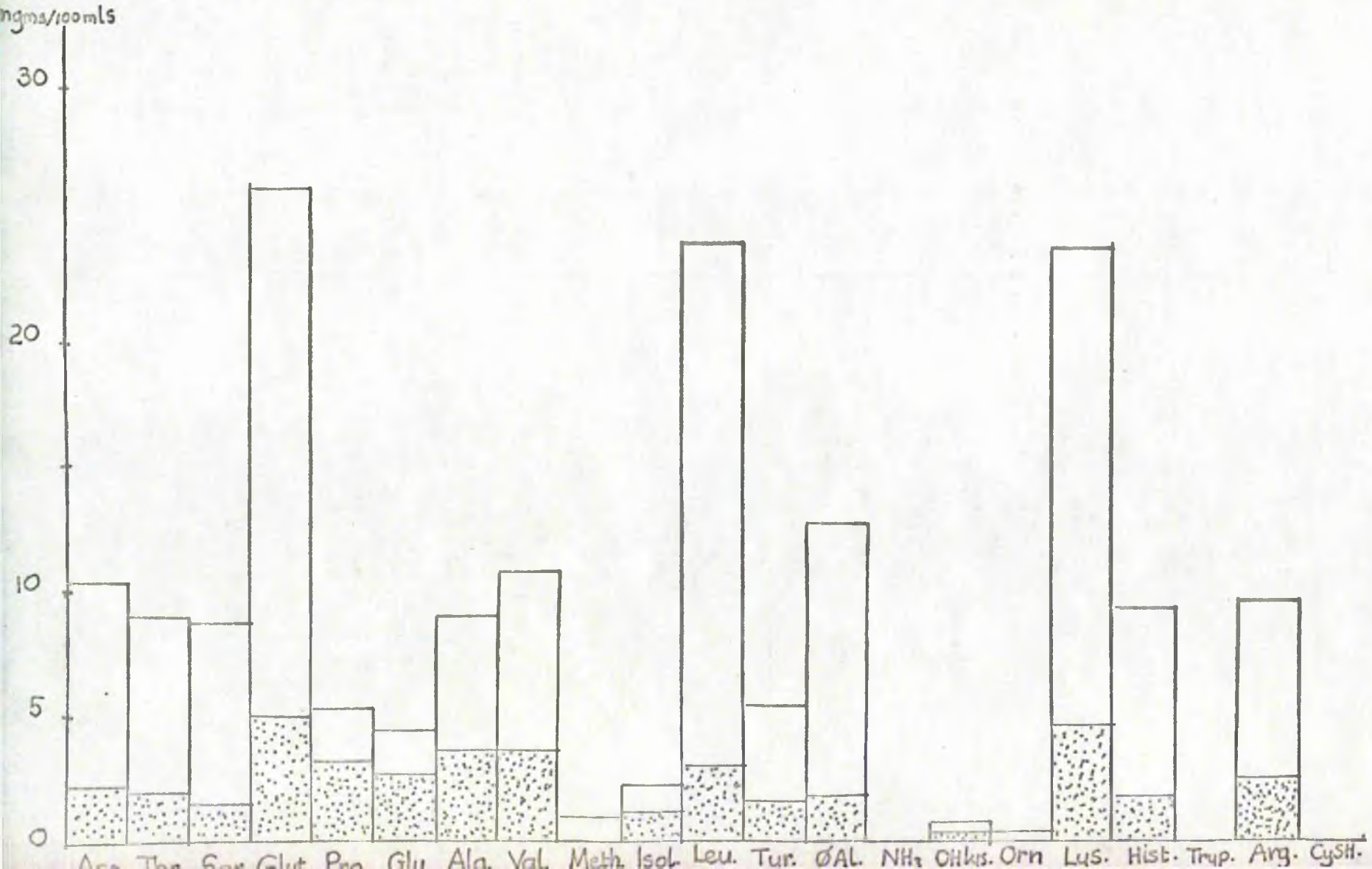
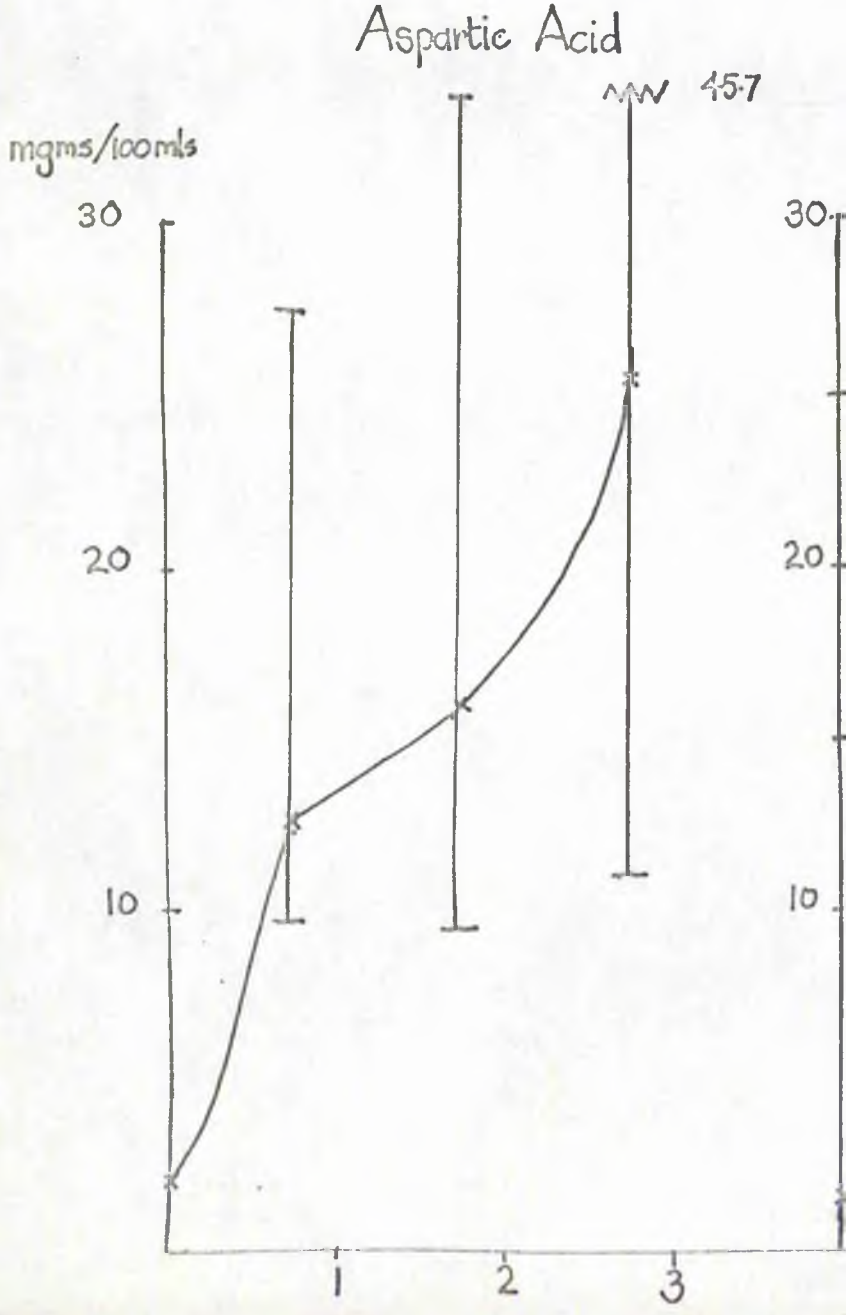


FIG. 10.18 Plasma



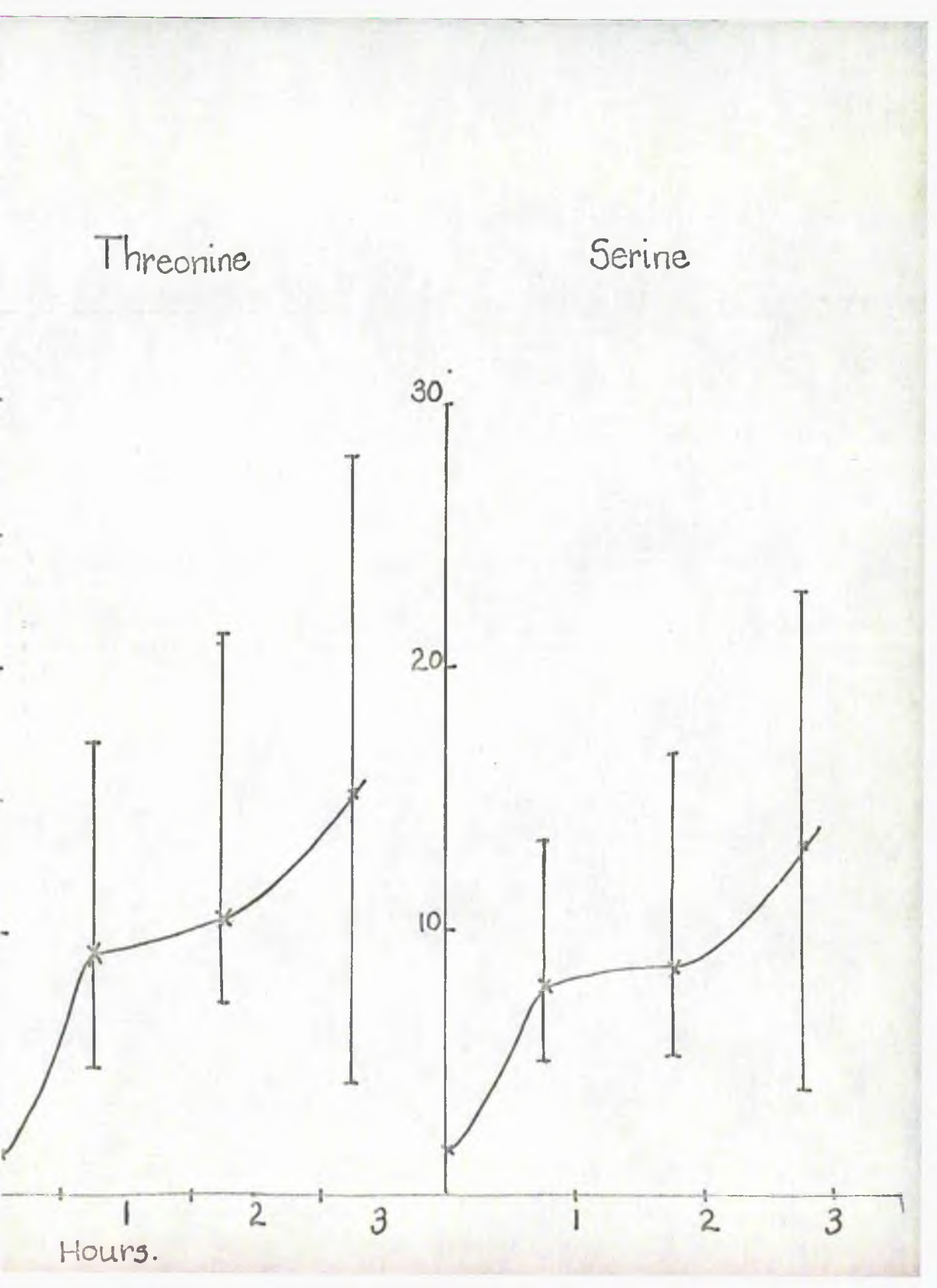

Threonine
Serine
30
20
10
1
2
3
1
2
3
Hours.

FIG. 10.19 Plasma

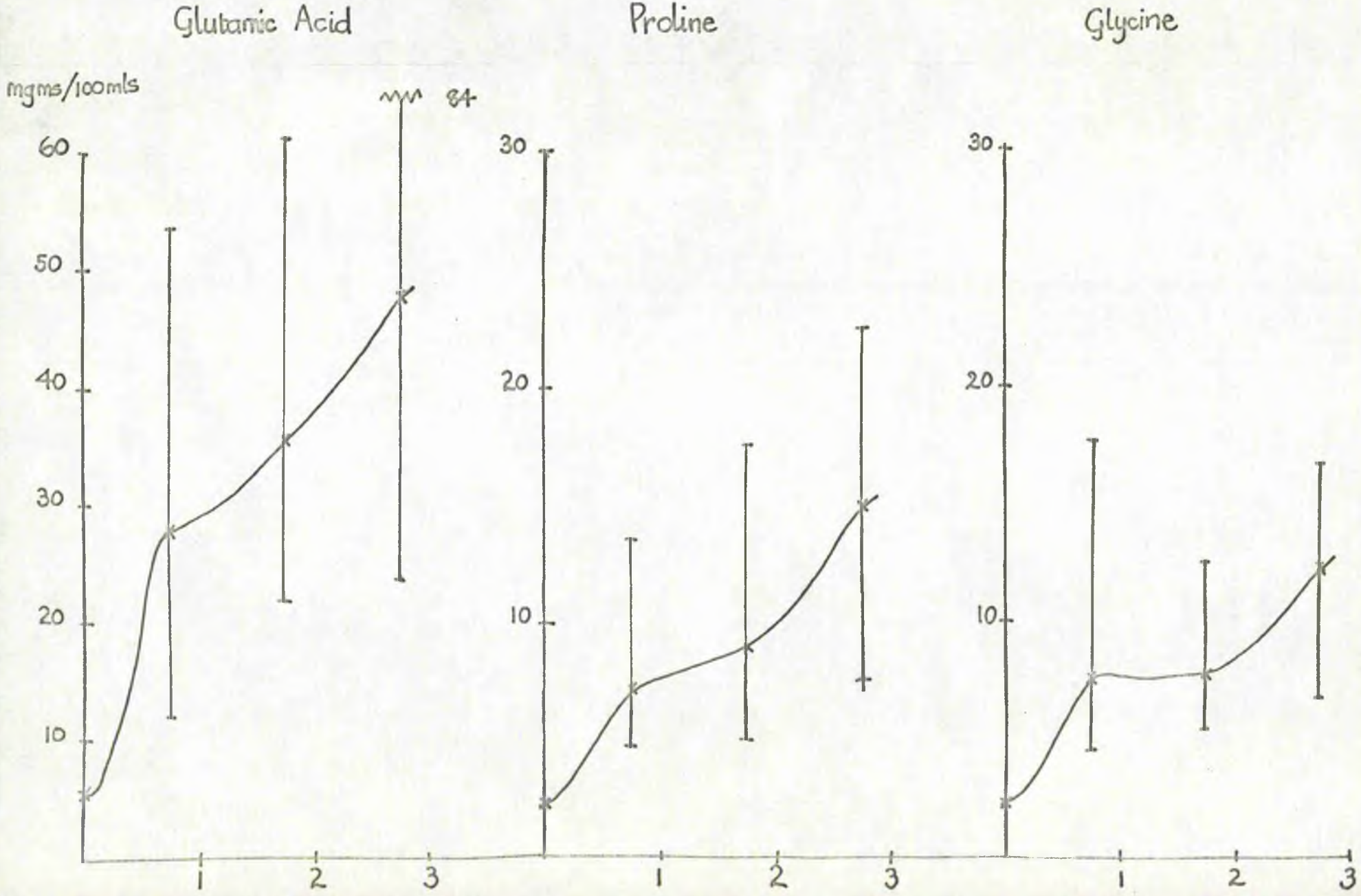


FIG. 10·20 Plasma

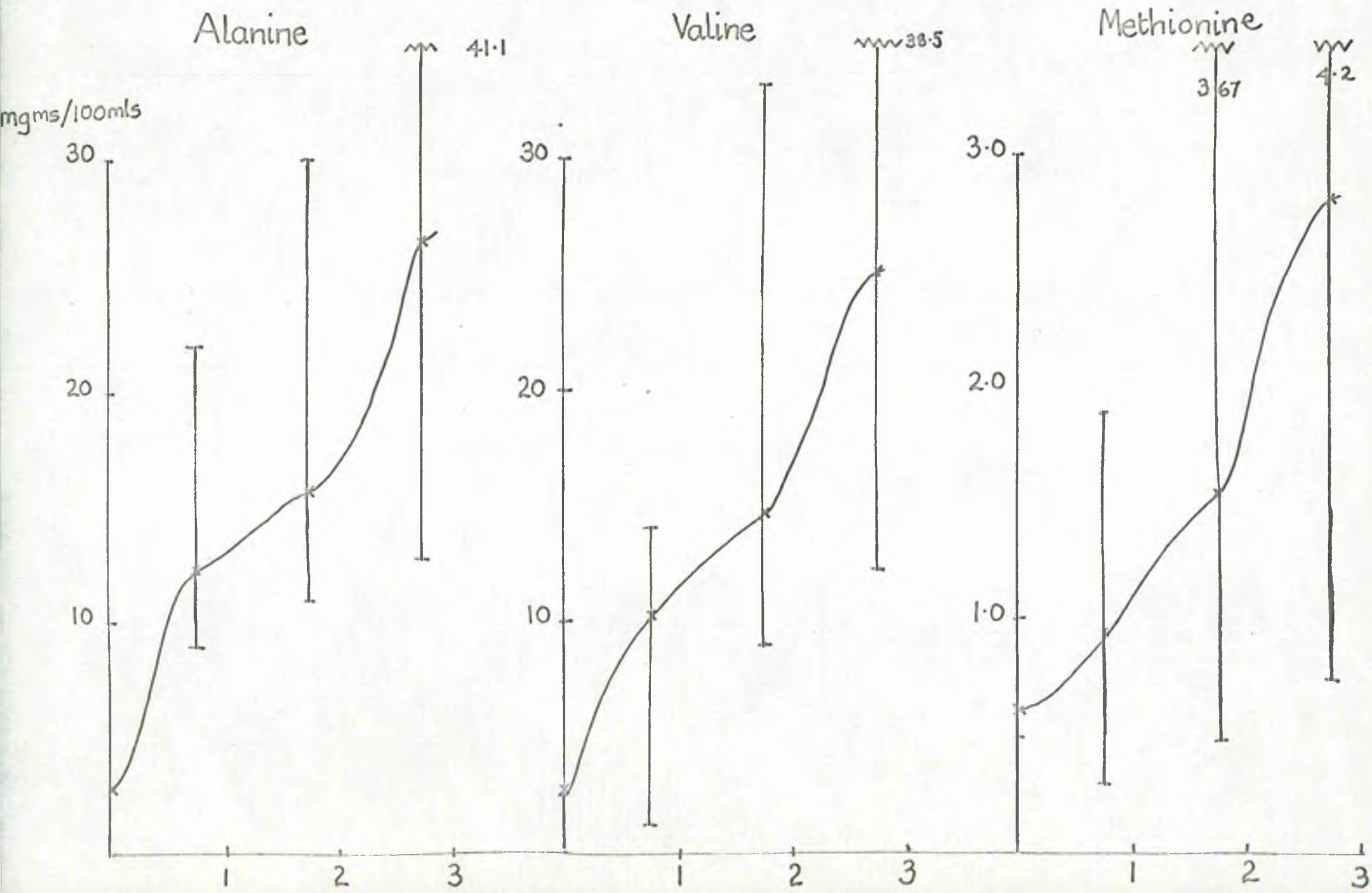


FIG. 10·21. Plasma

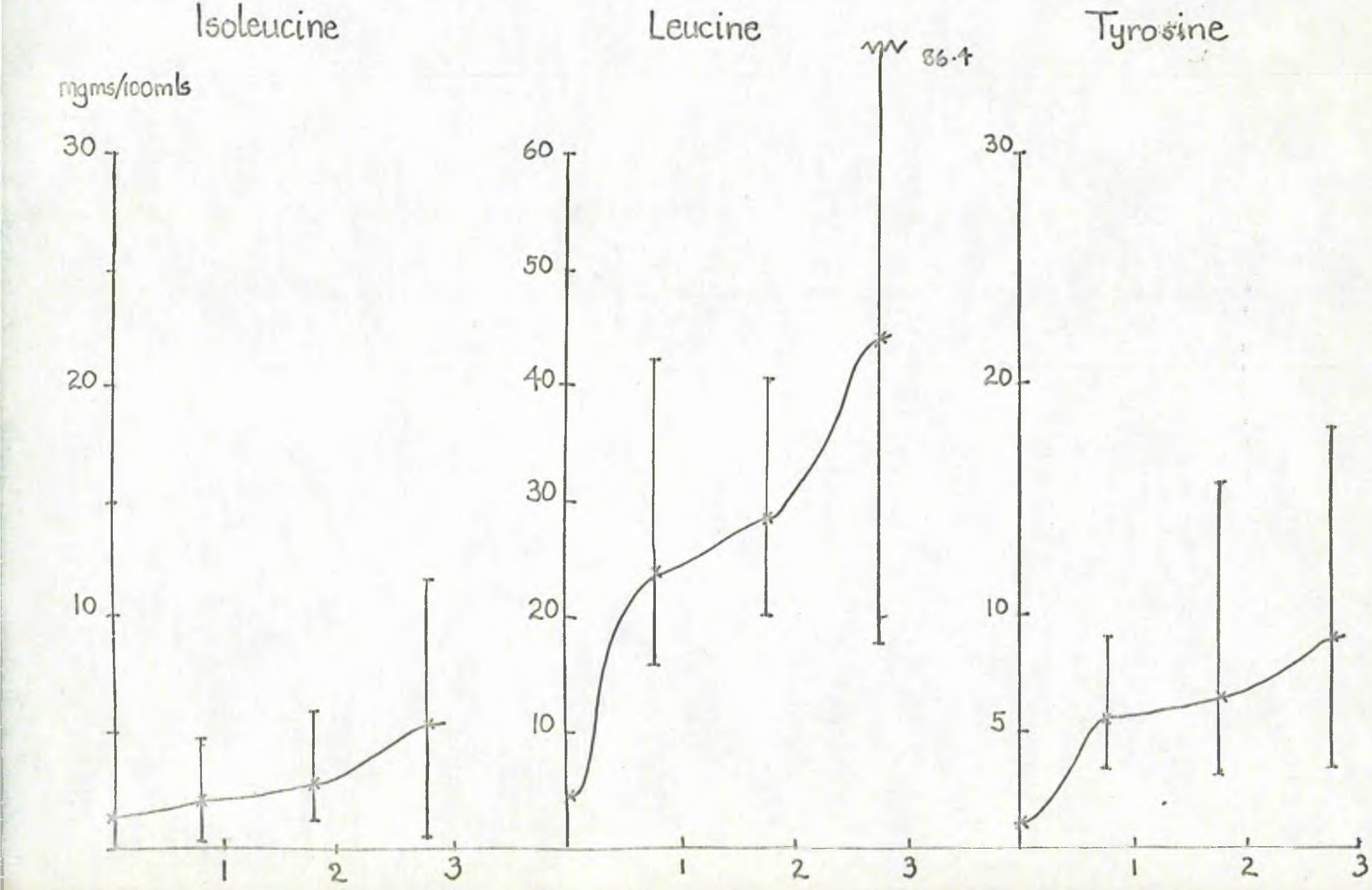


FIG. 10·22 Plasma

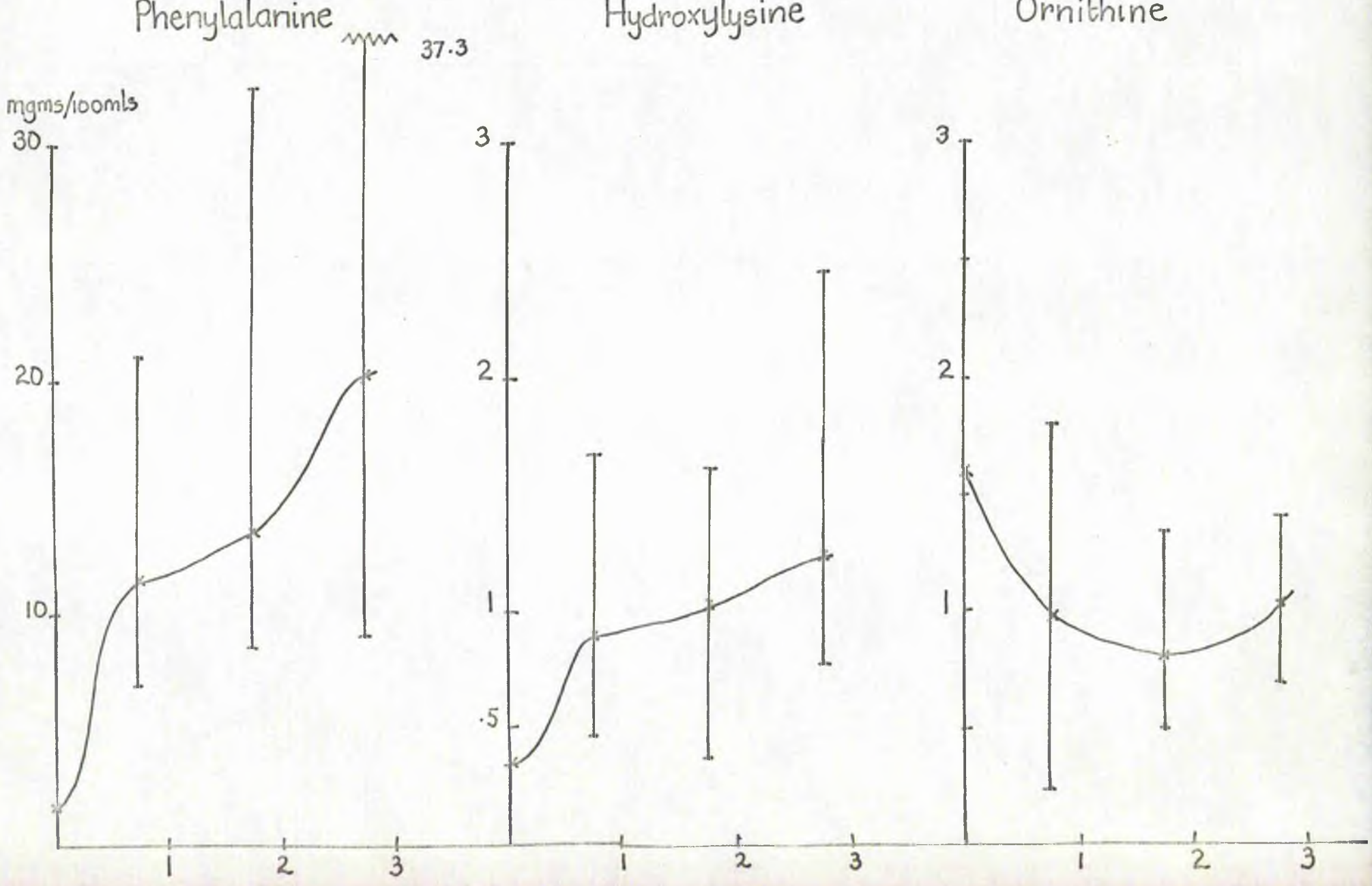
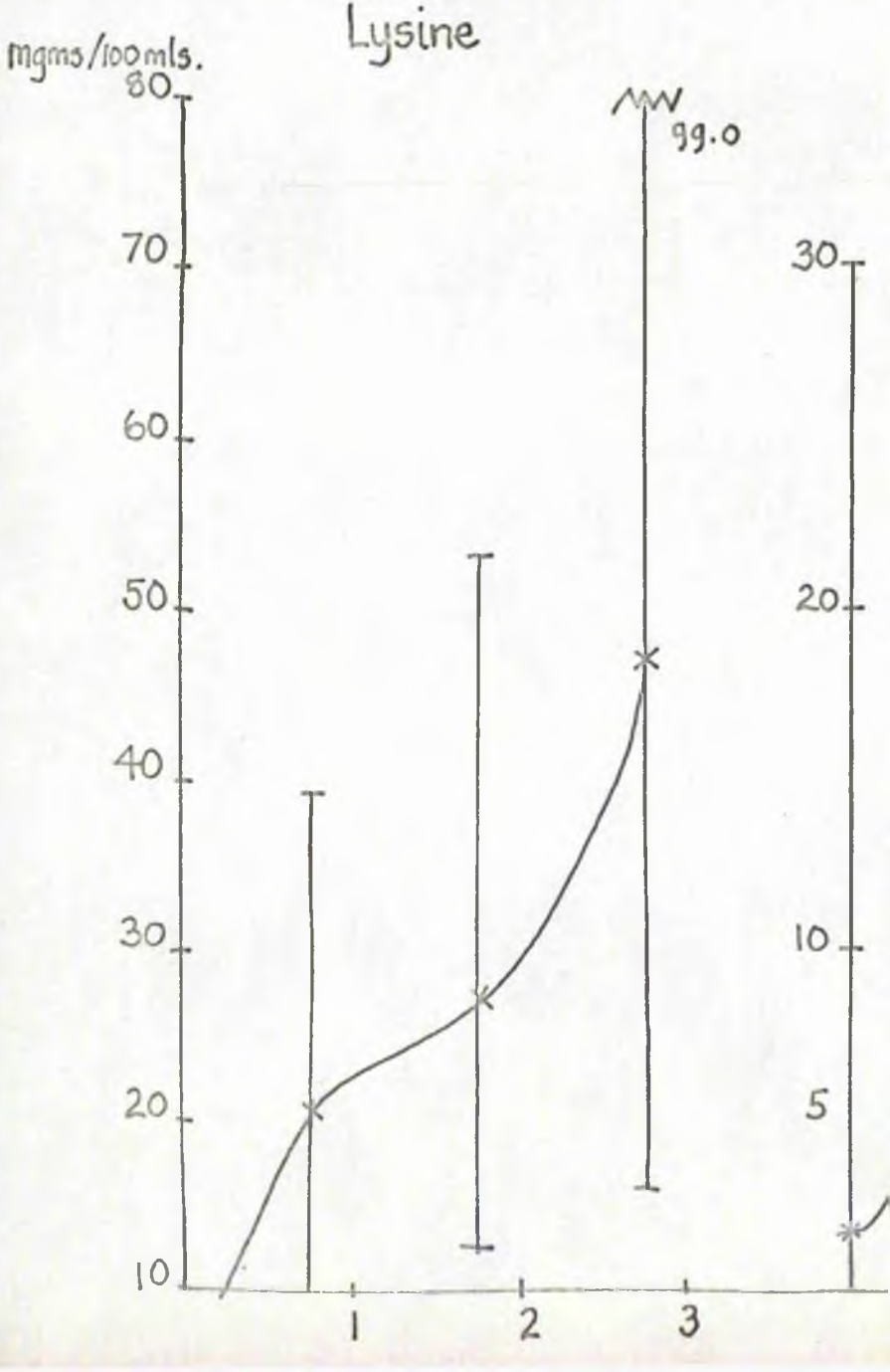


FIG. 10·23 Plasma



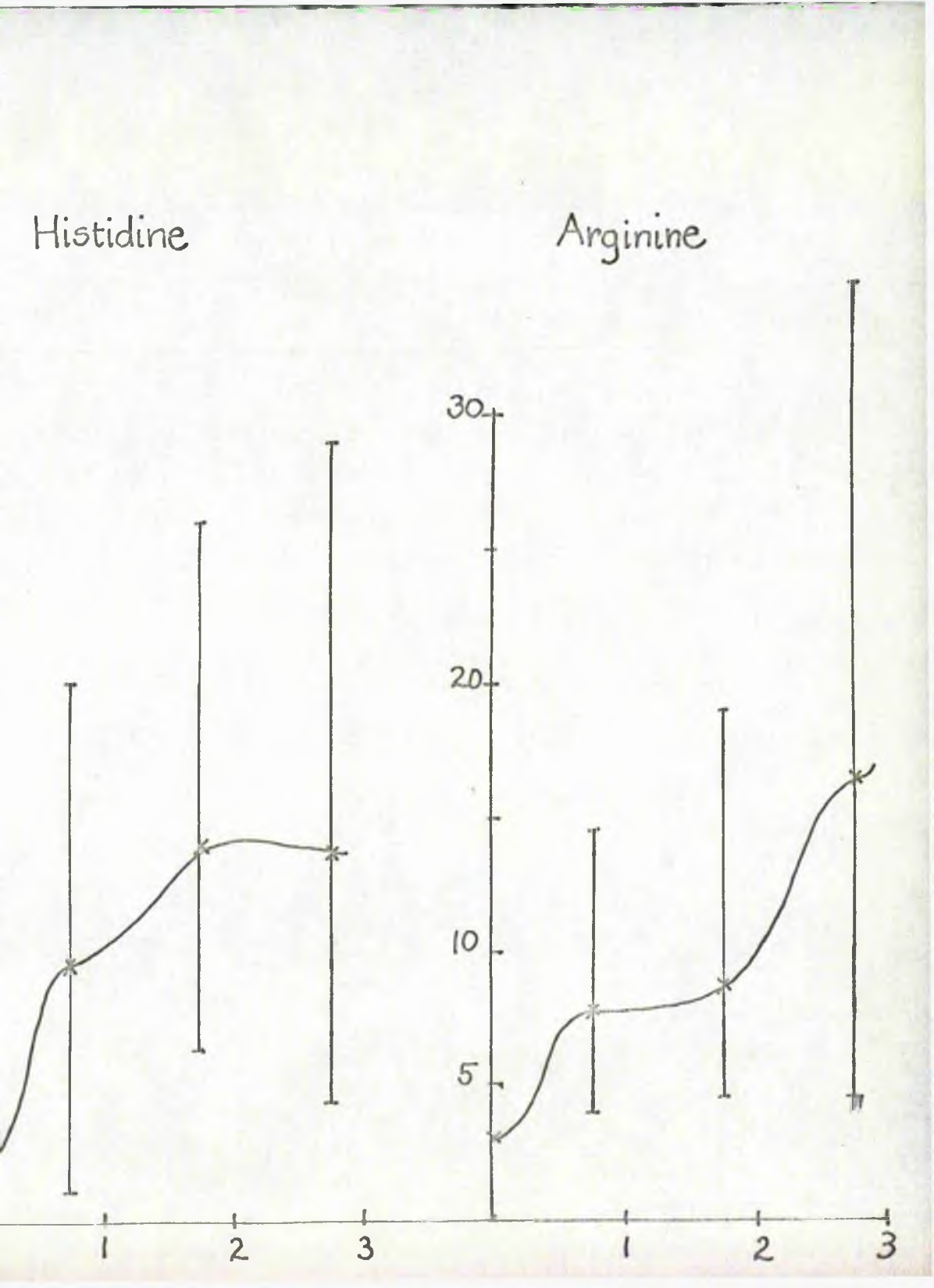

Histidine
Arginine
30
20
10
5
1
2
3
1
2
3

FIG. 10·24 Plasma

Cysteine

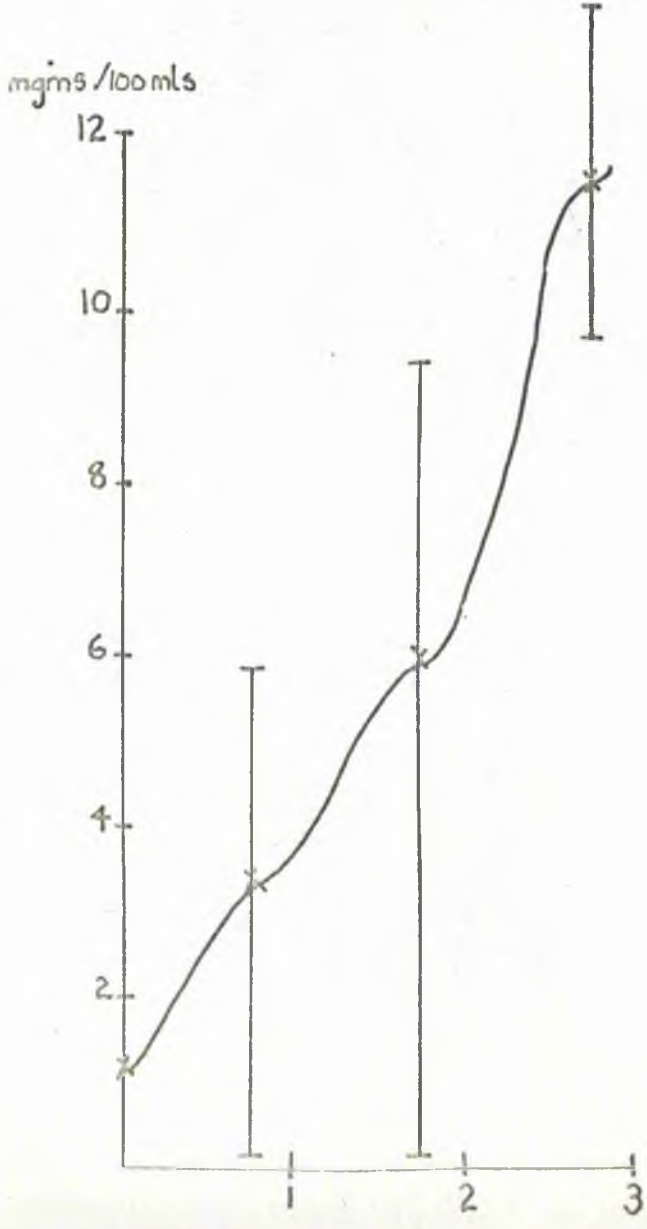


FIG. 10·25 Urinary Aspartic Acid.

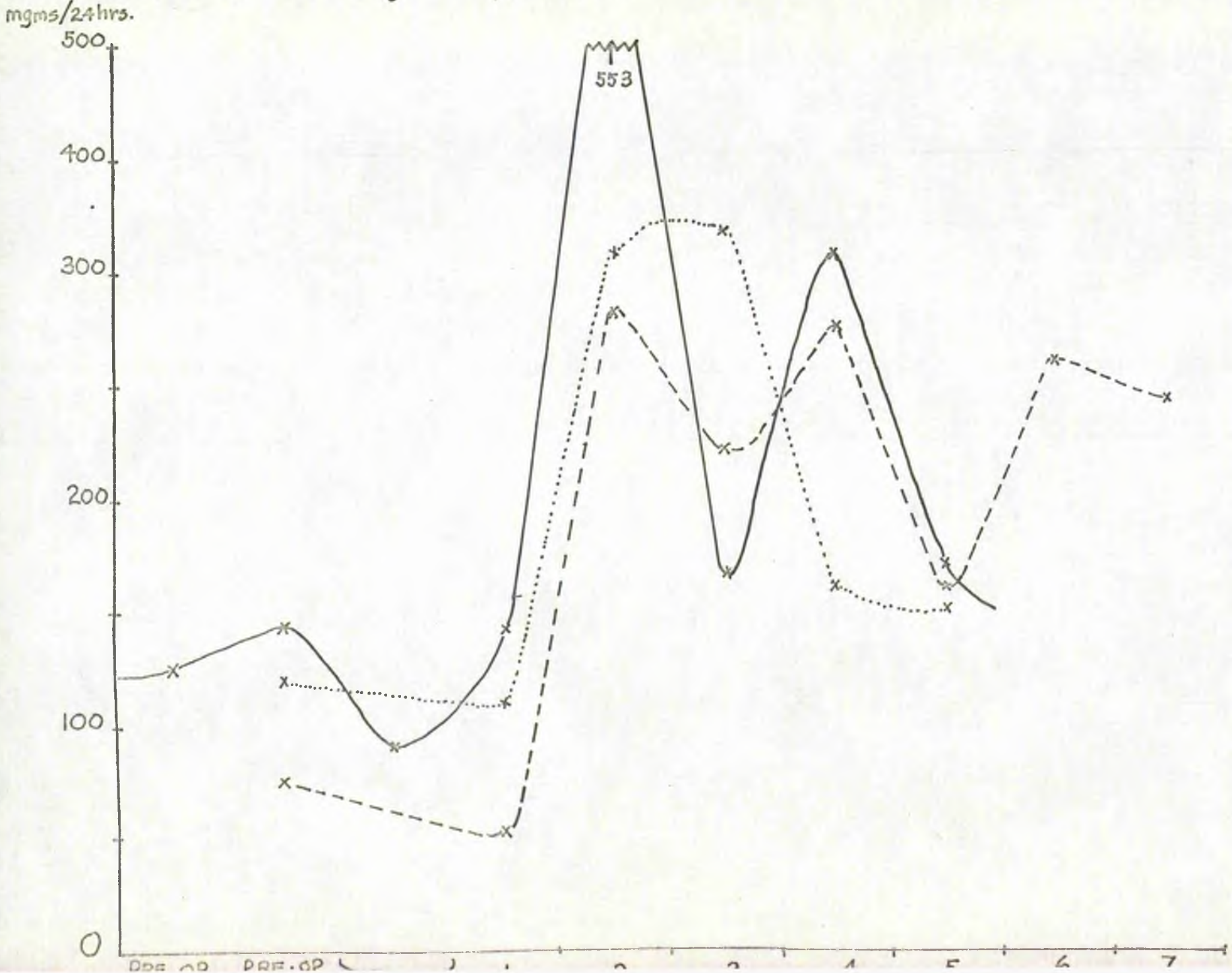


FIG. 10.26 Urinary Threonine

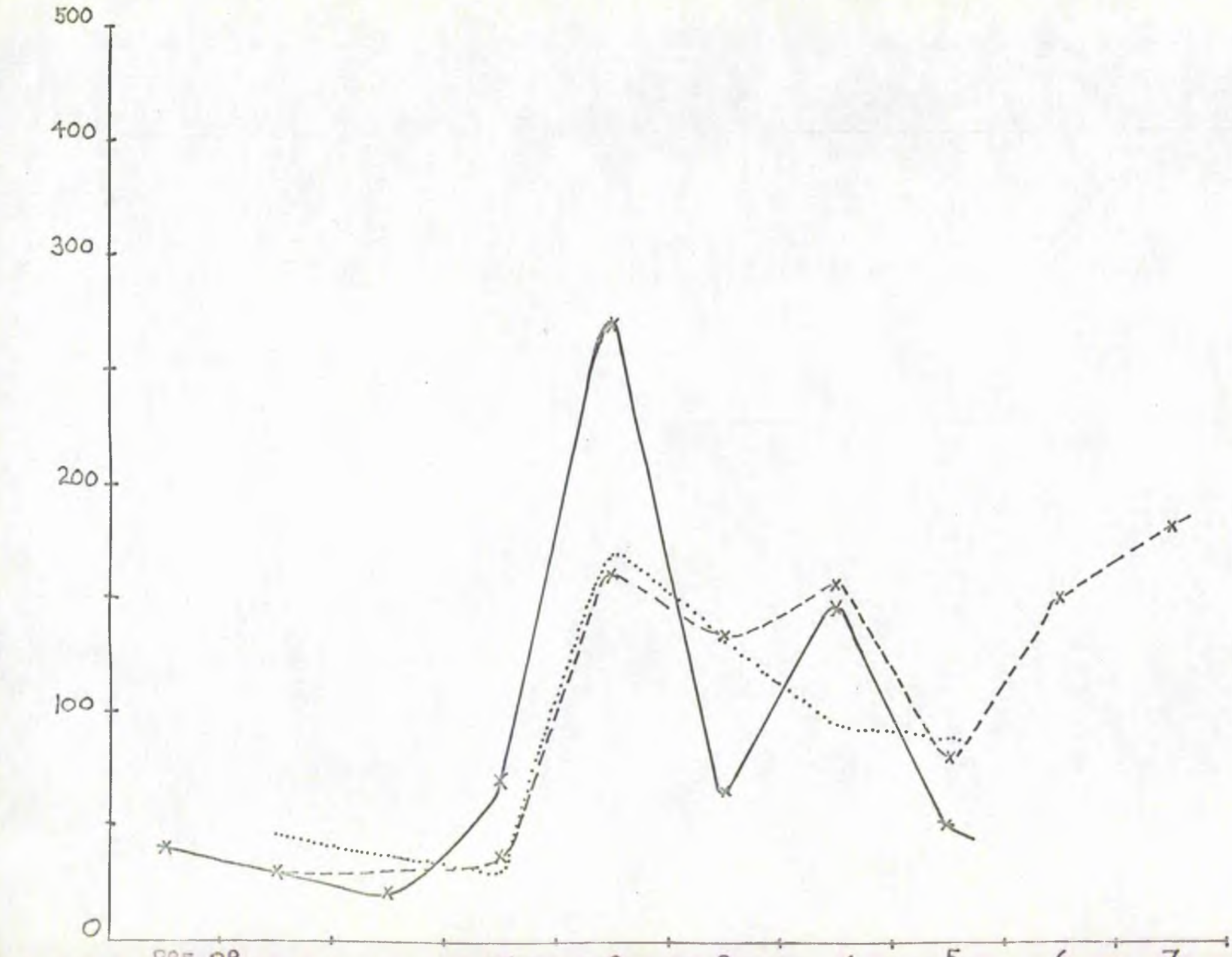


FIG. 10·27 Urinary Serine

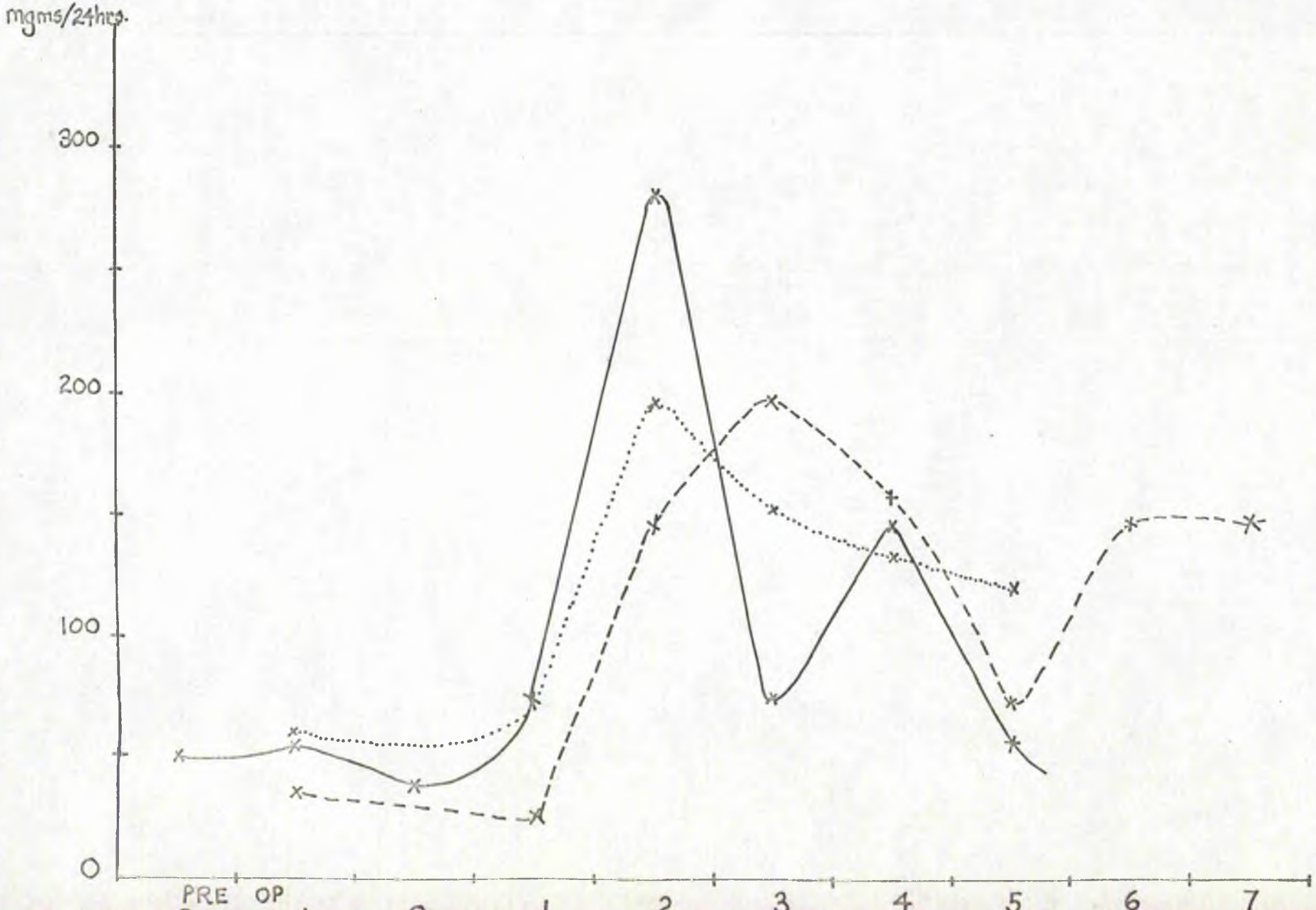


FIG. 10·28 Urinary Glutamic Acid.

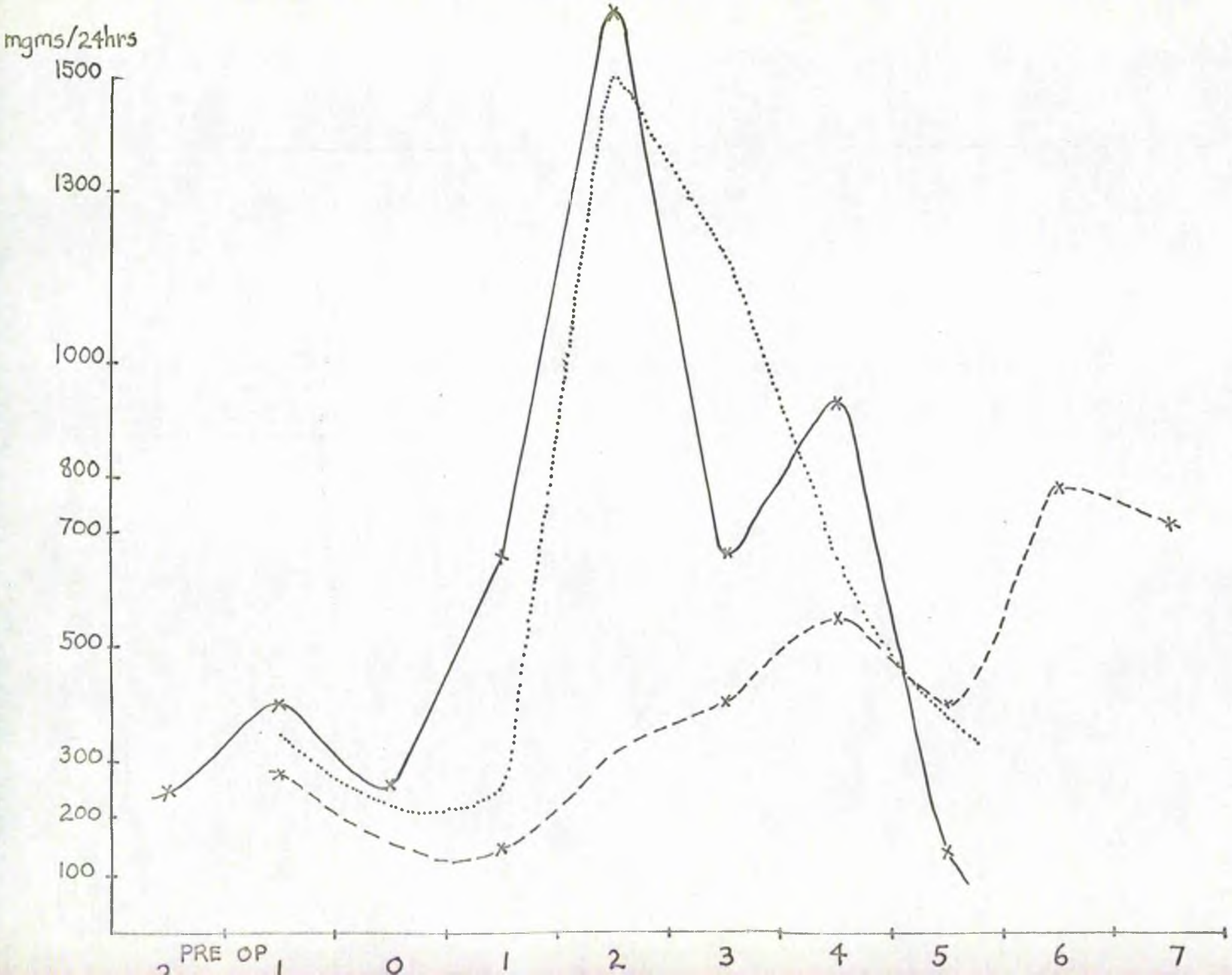


FIG. 10.29 Urinary Proline

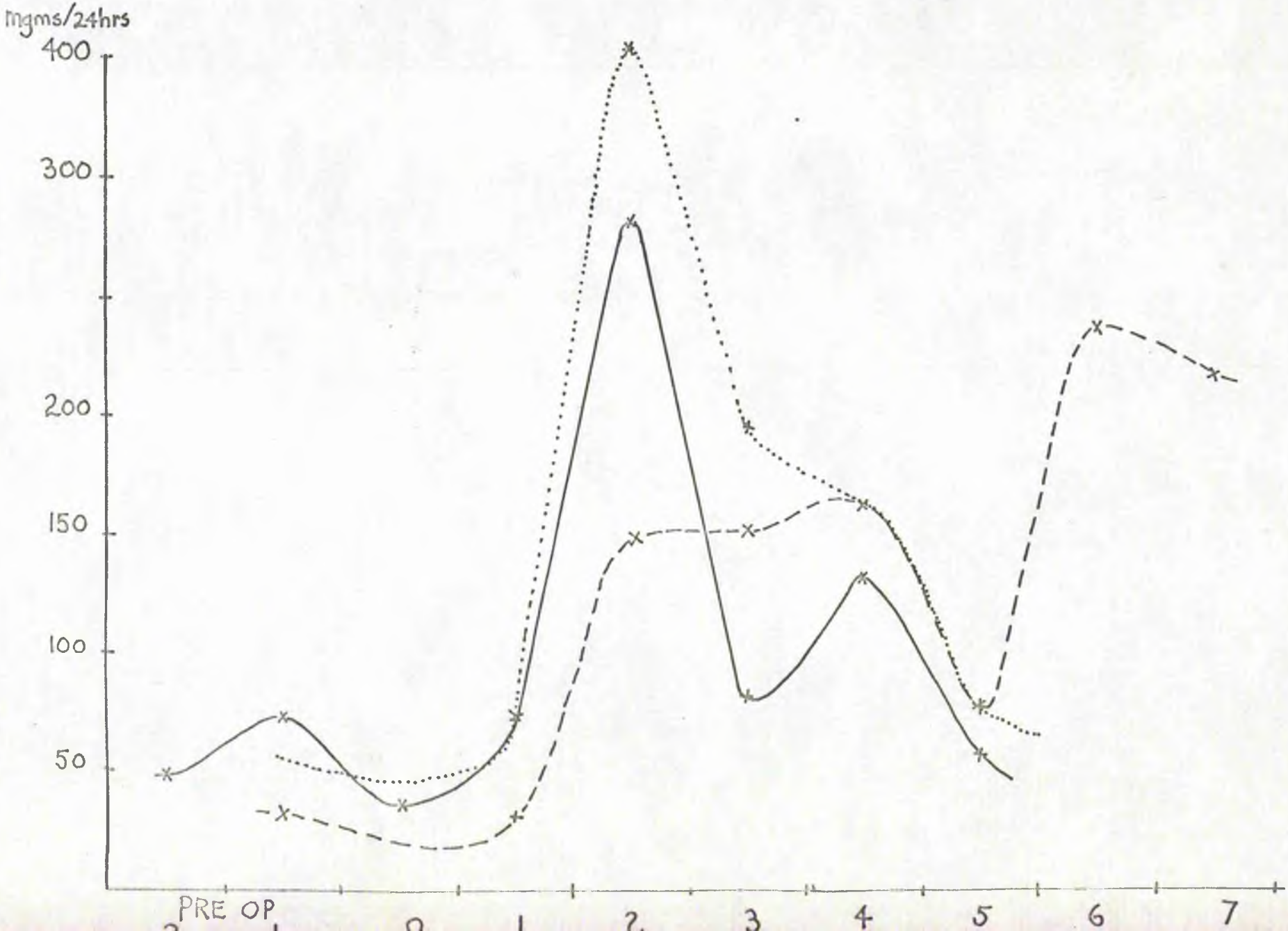


FIG. 10·30 Urinary Glycine

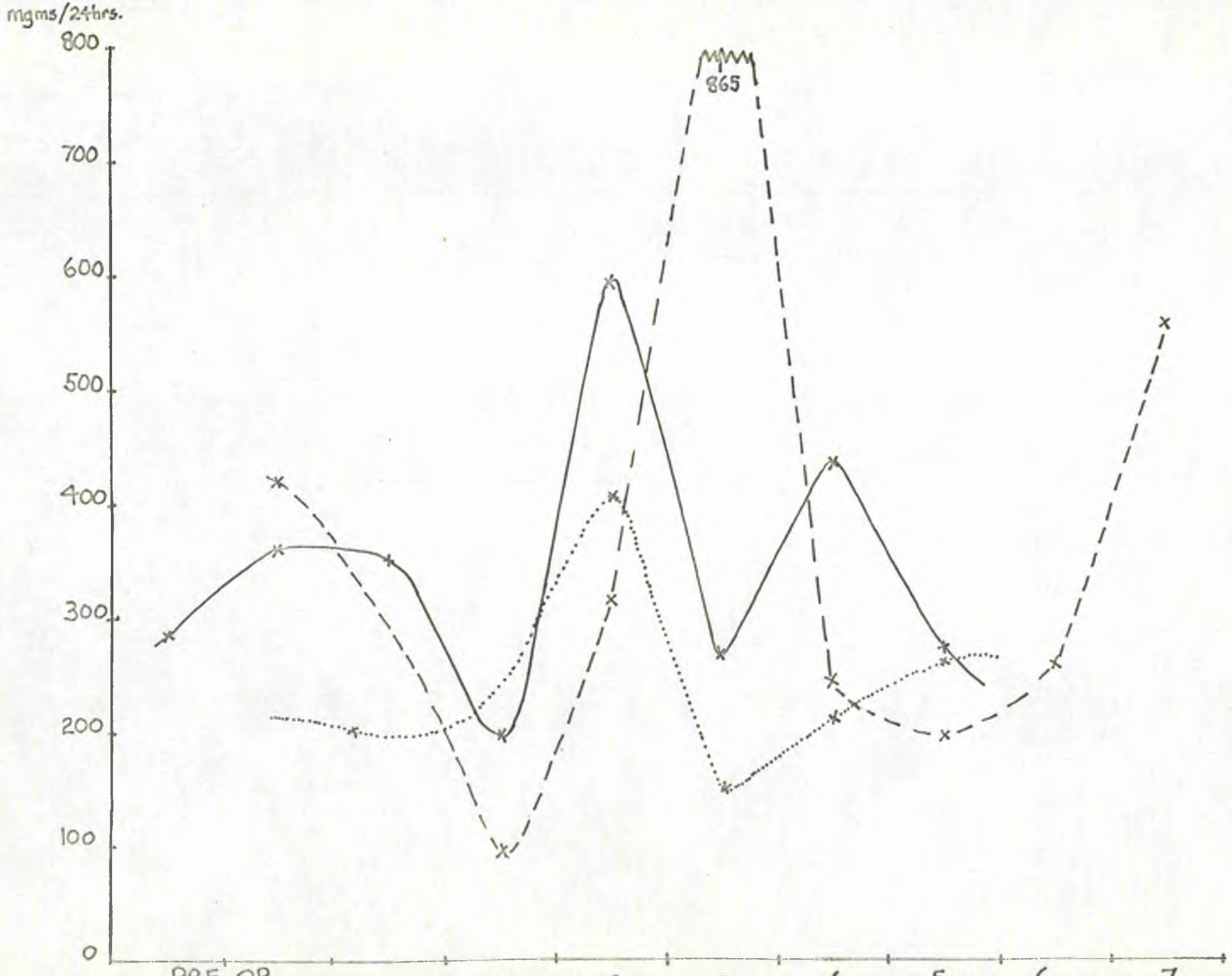


FIG. 10·31 Urinary Alanine

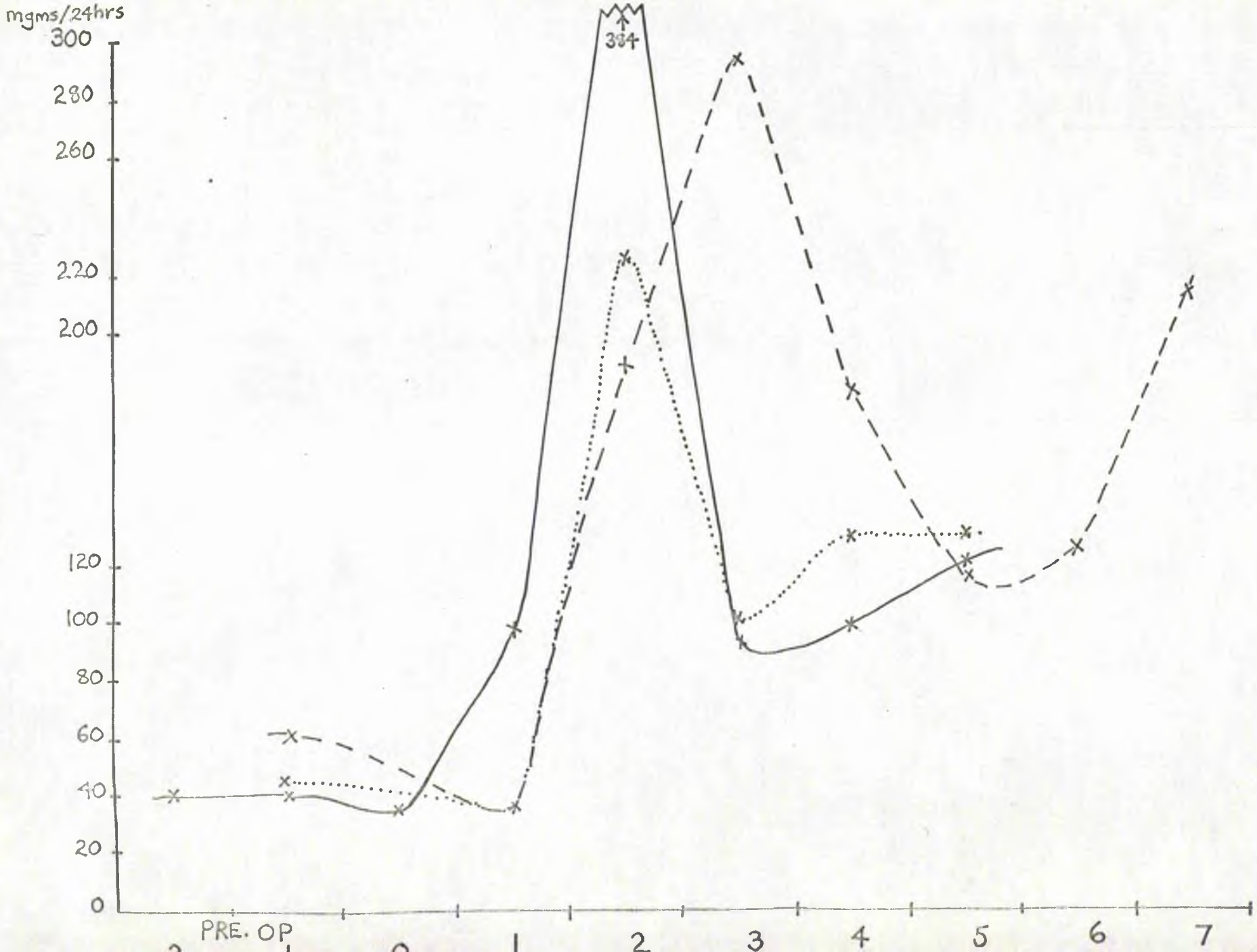


FIG. 10.32. Urinary Valine

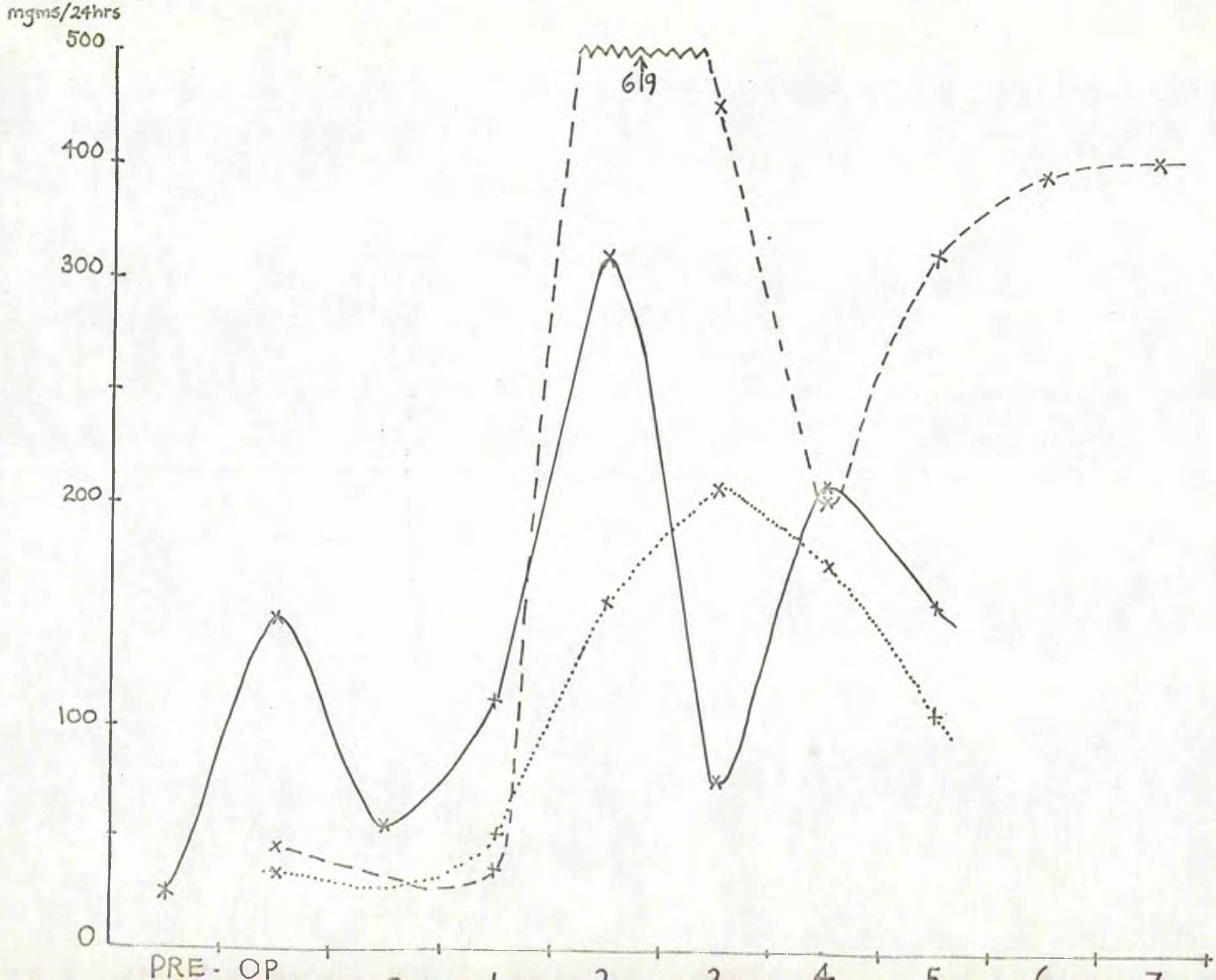


FIG. 10.33 Urinary Cysteine

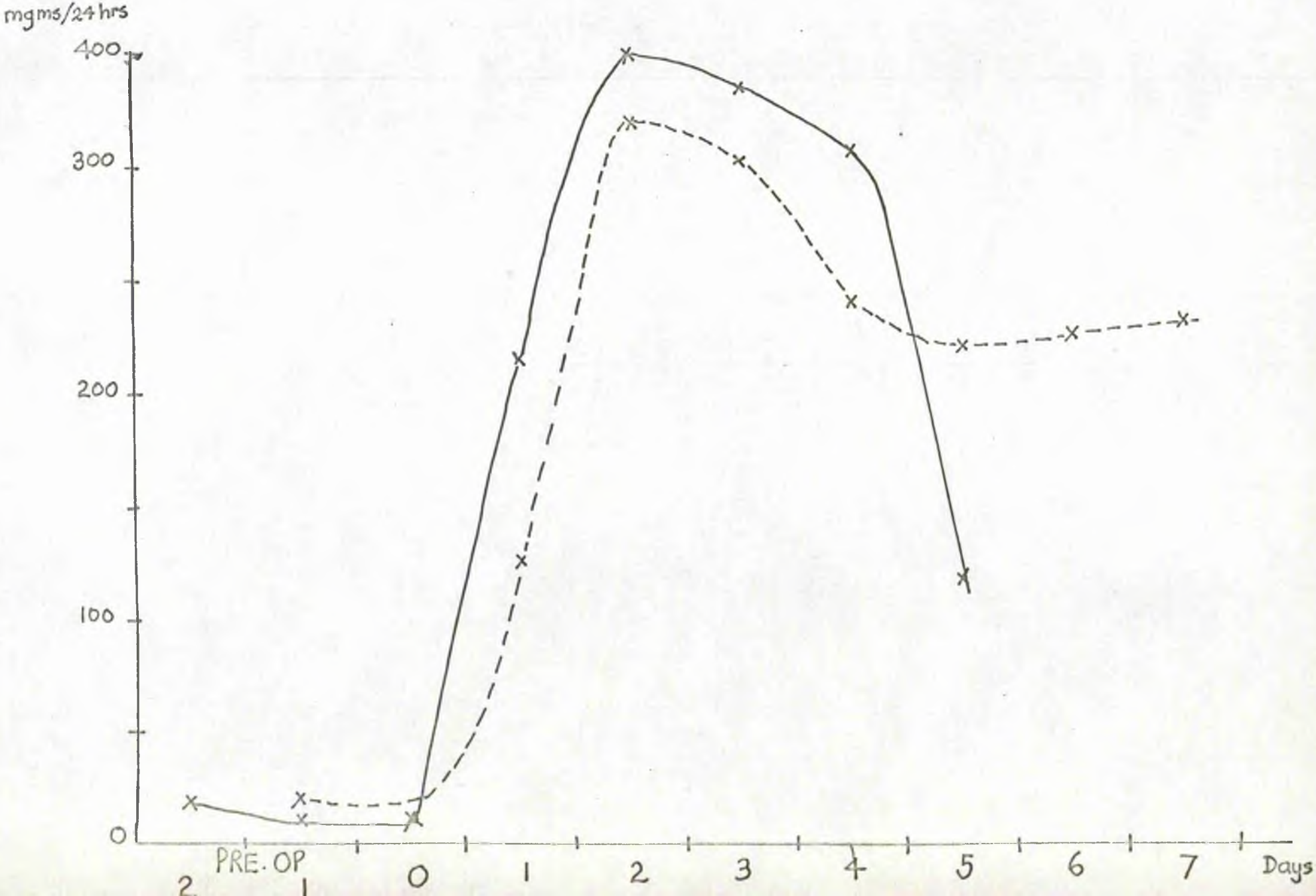


FIG. 10.34 Urinary Methionine

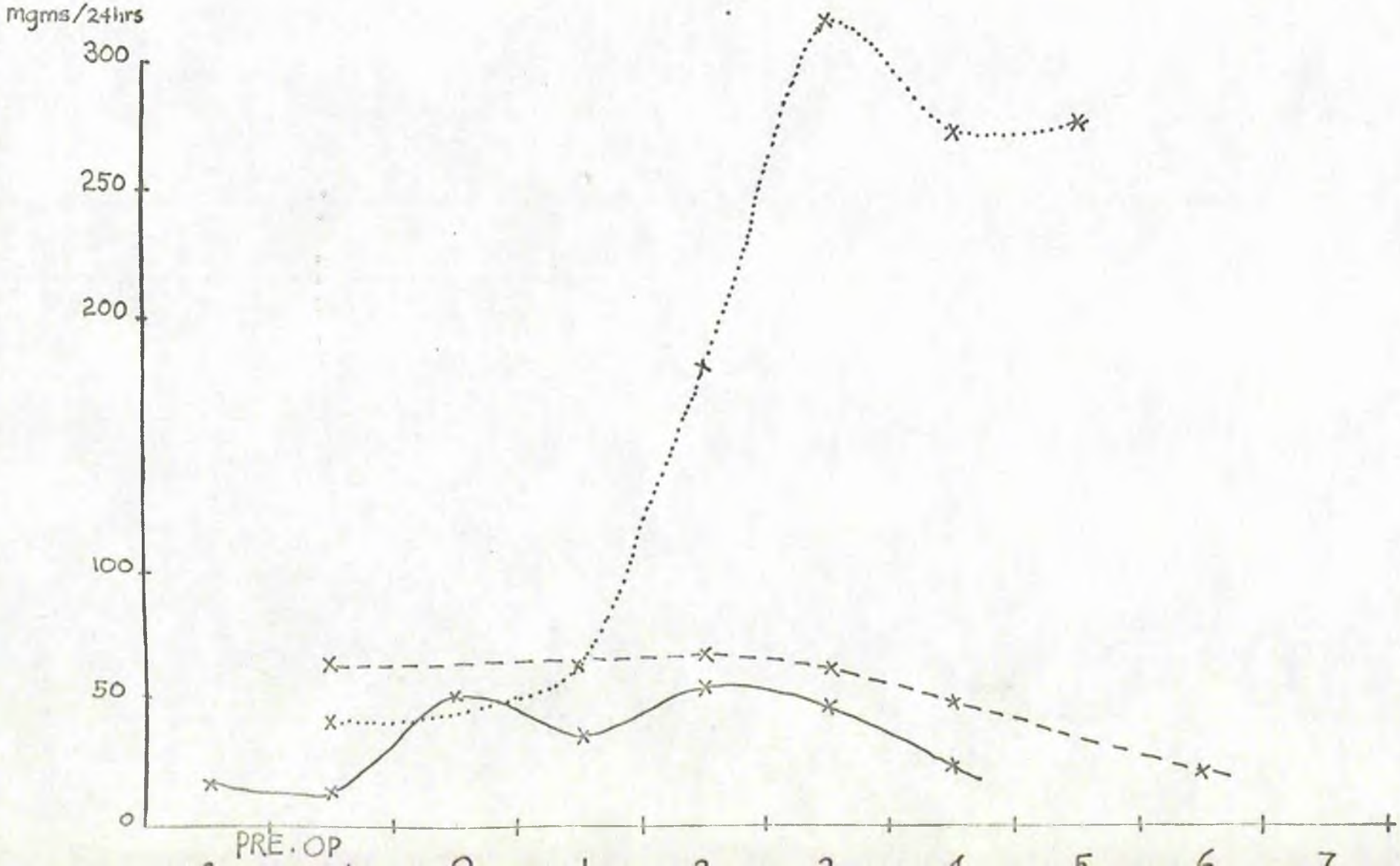


FIG. 10.35. Urinary Isoleucine

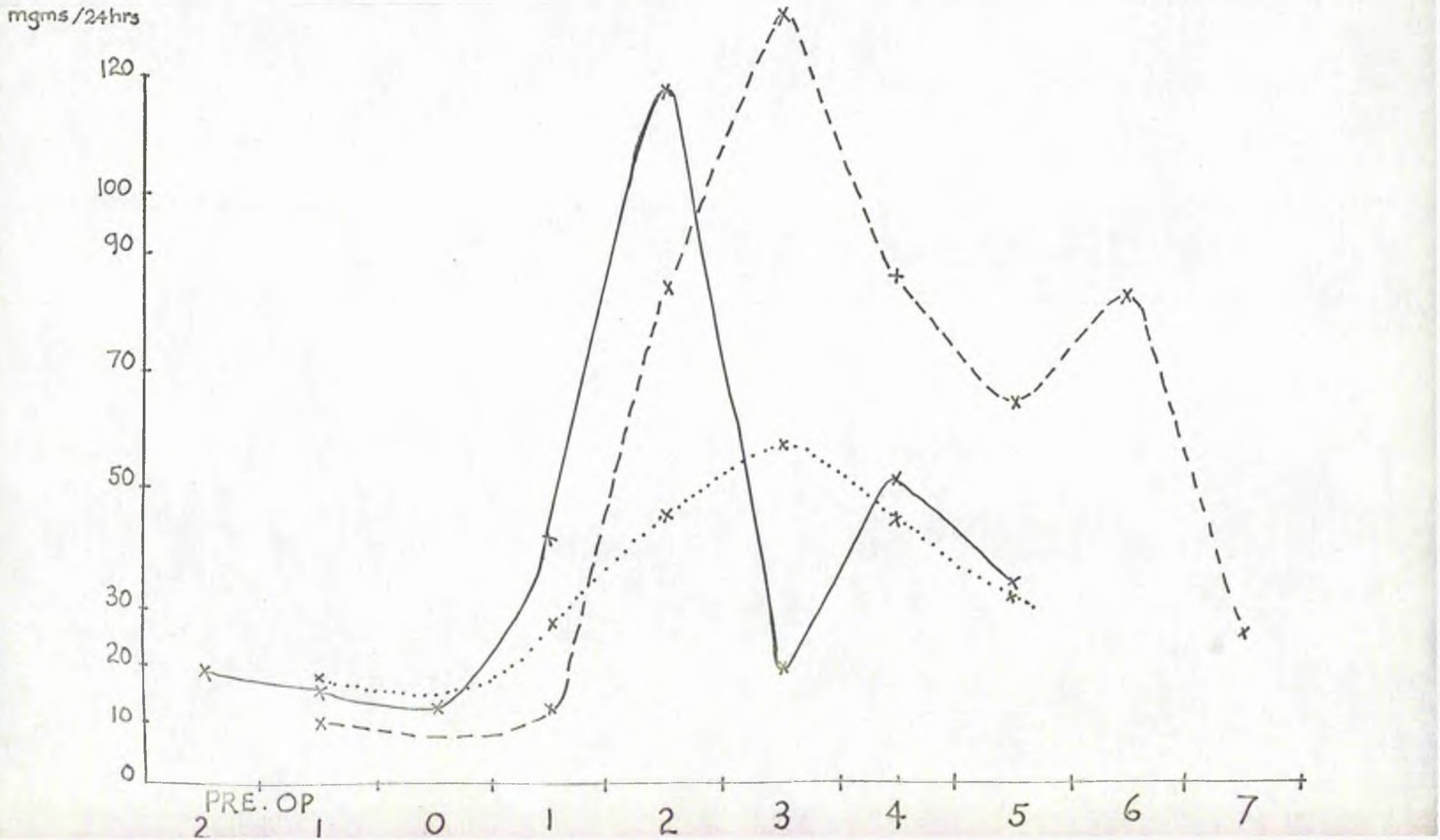


FIG. 10.36 Urinary Leucine

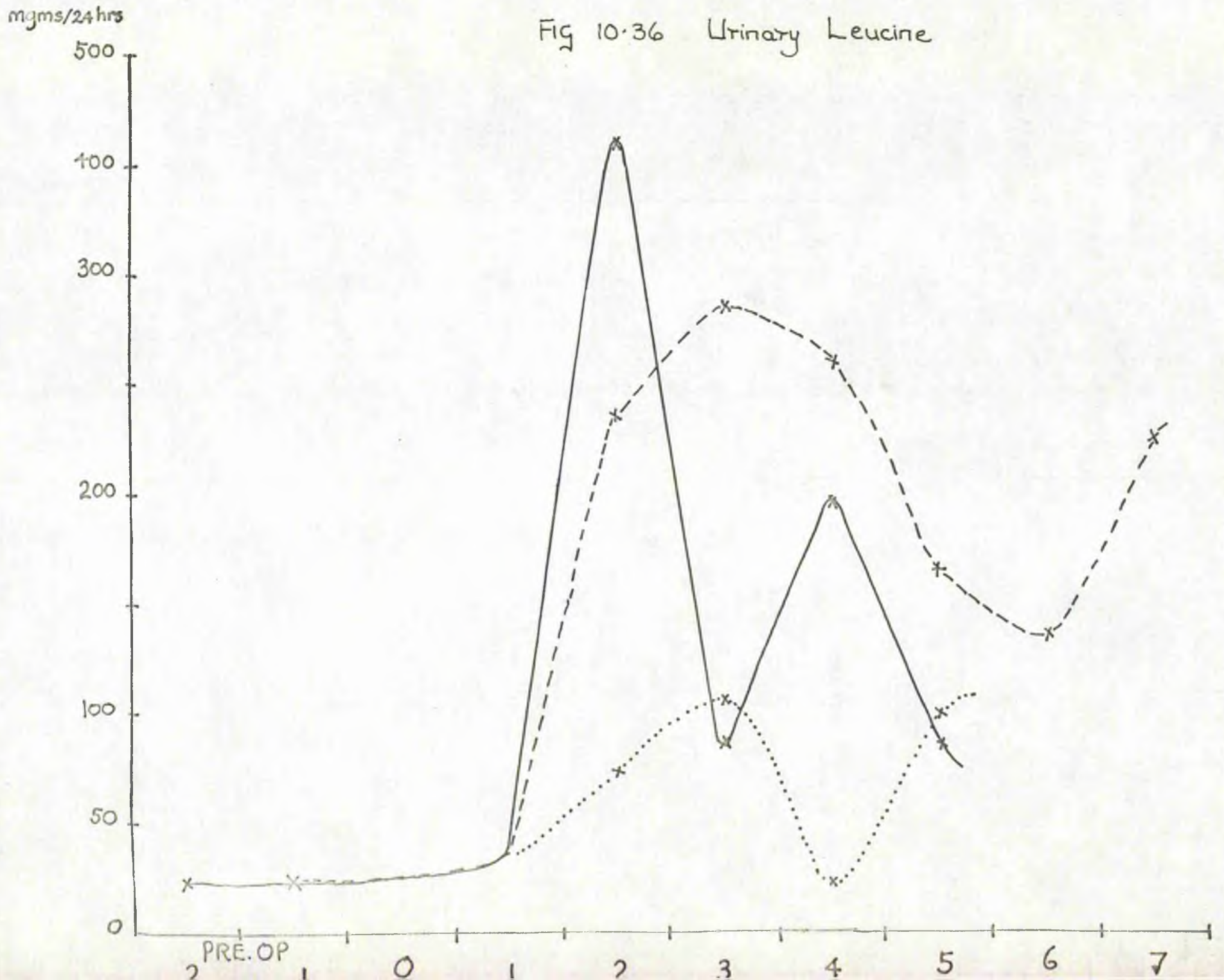


Fig 10·36 Urinary Leucine

FIG. 10-37 Urinary Tyrosine

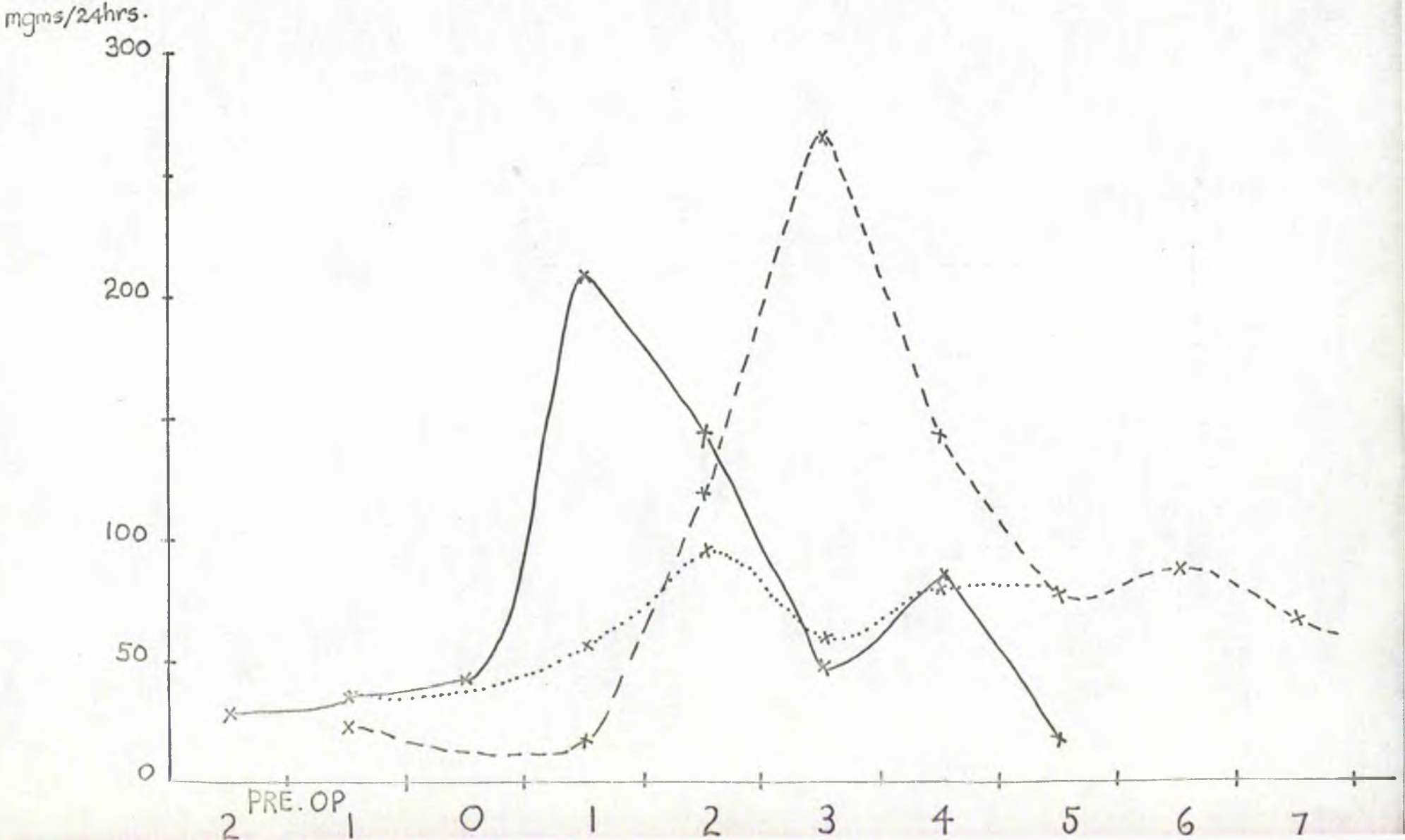


FIG. 10.38 Urinary Phenyl Alanine

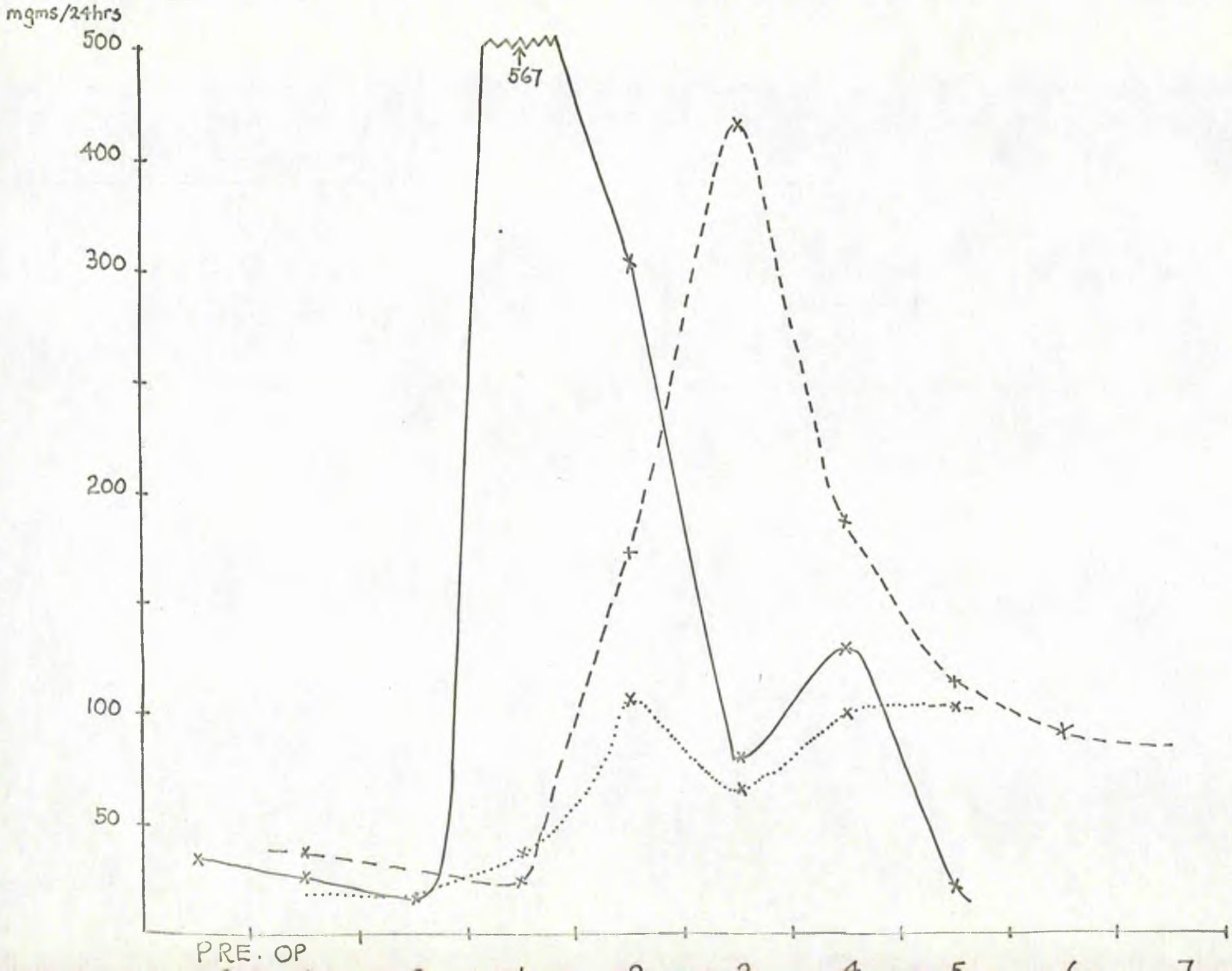


FIG. 10.40 Urinary Ornithine

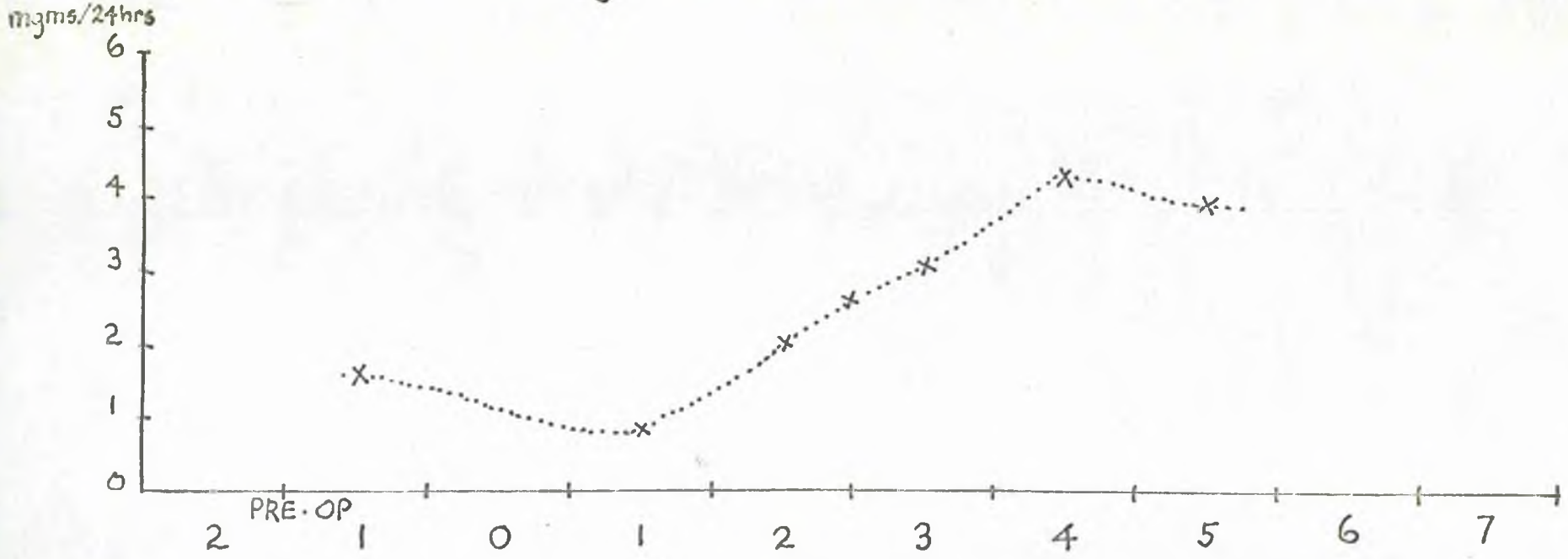


FIG. 10.39 Urinary Hydroxylysine

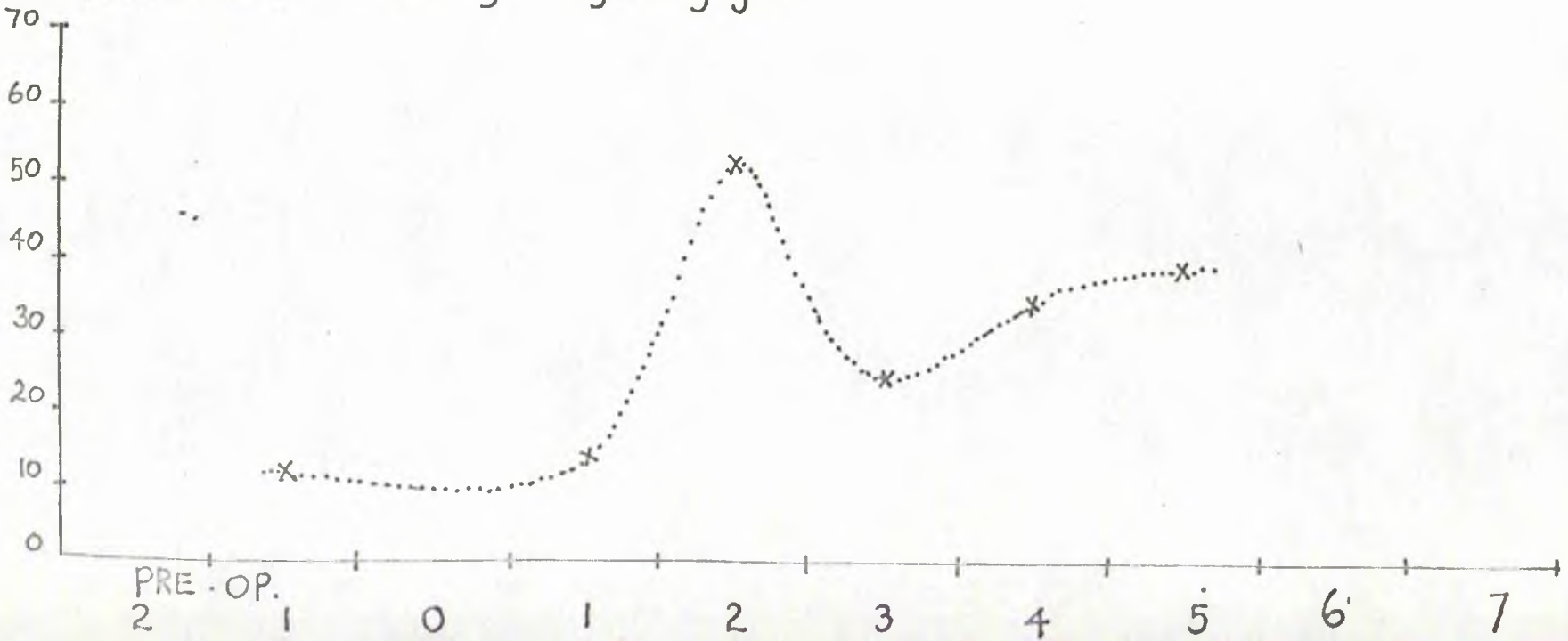


FIG. 10.41 Urinary Lysine

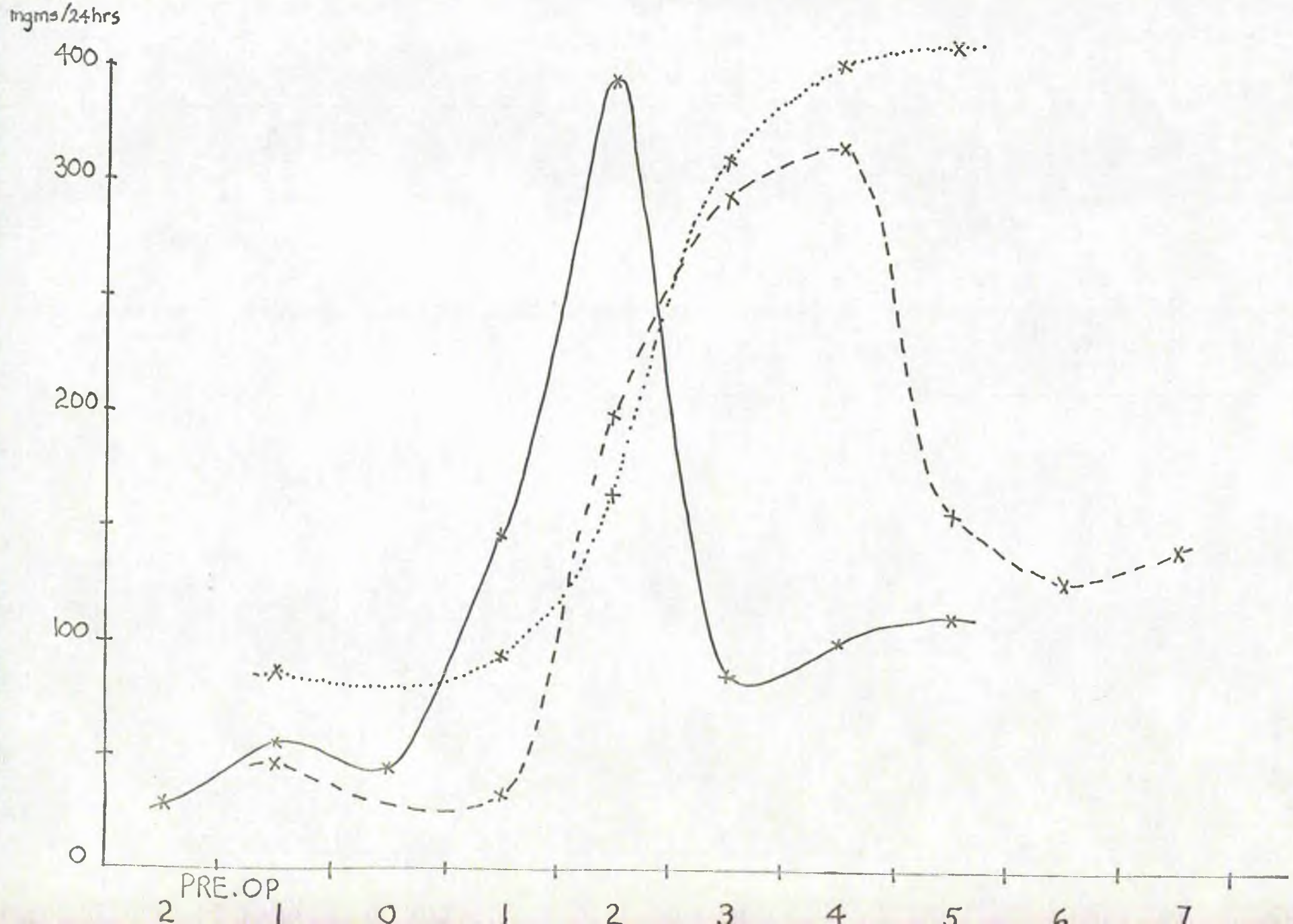


FIG. 10.42 Urinary Histidine

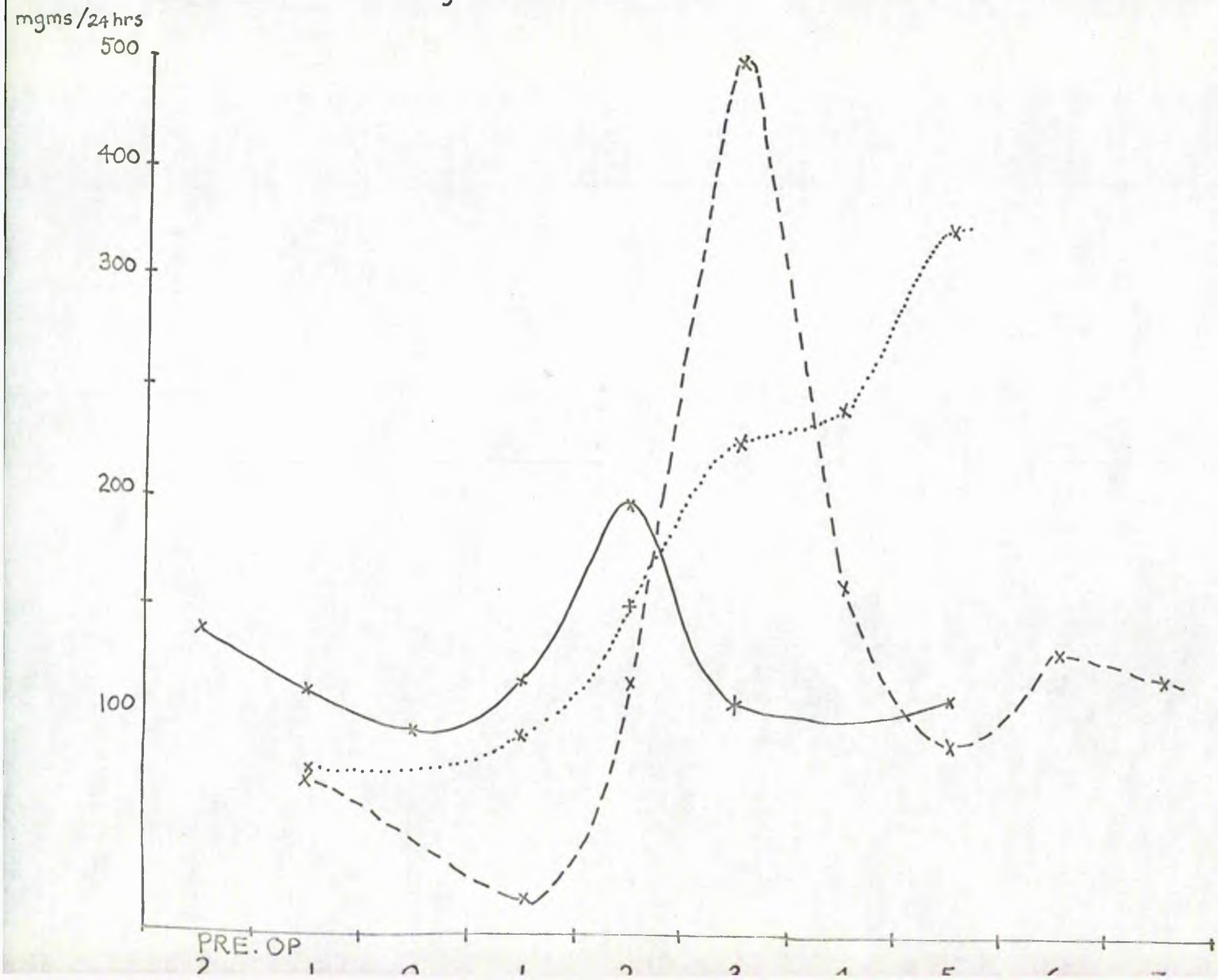
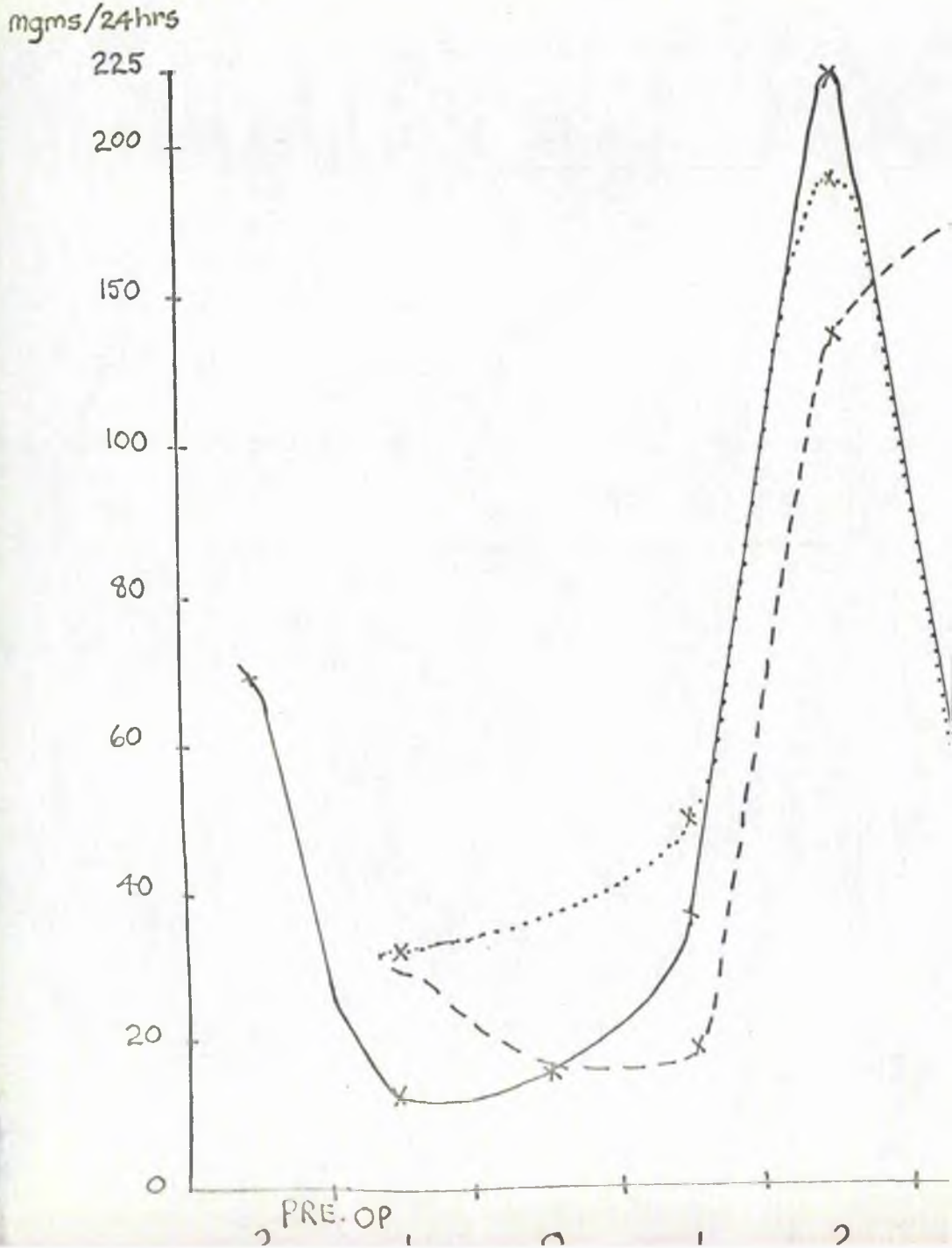
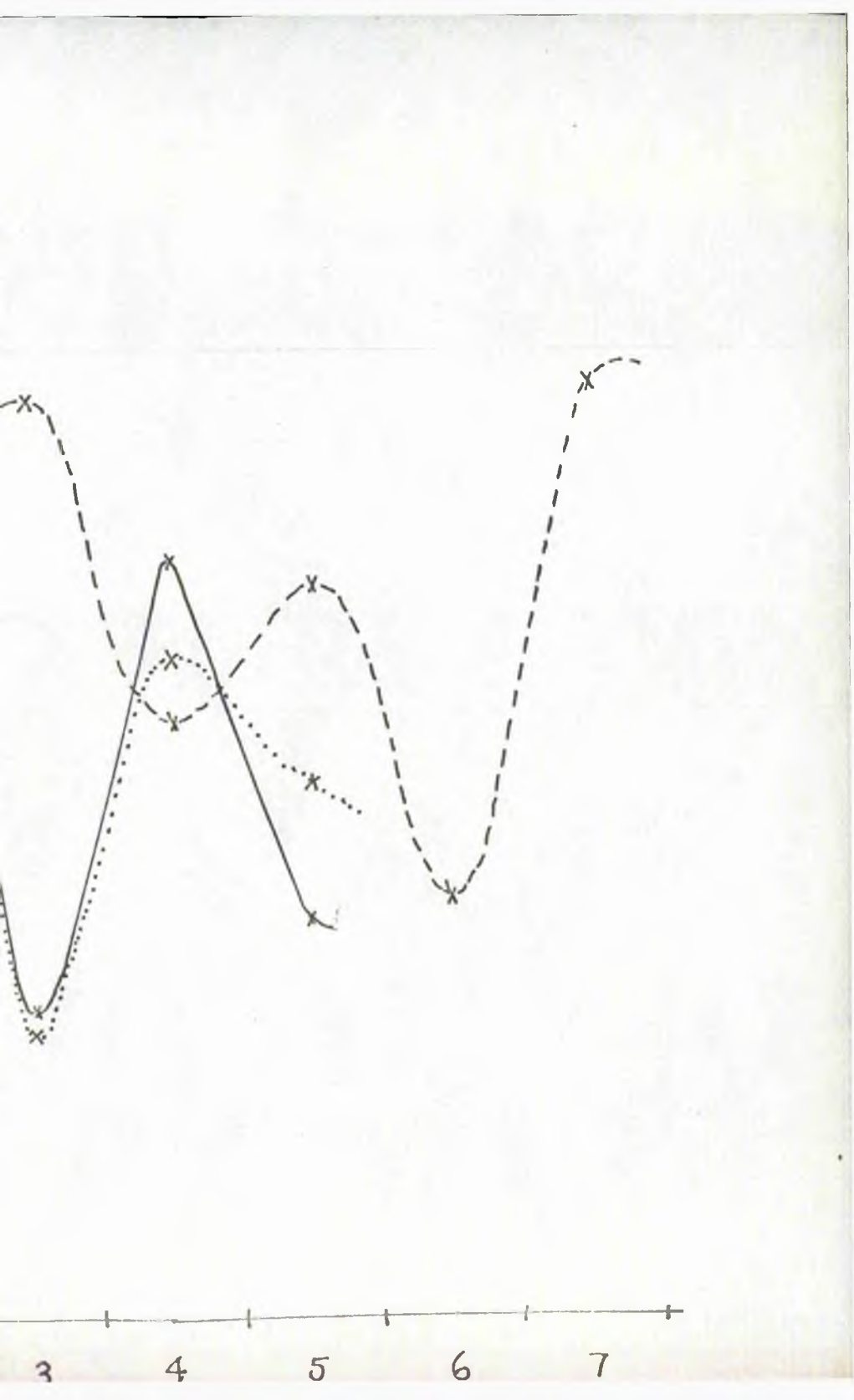
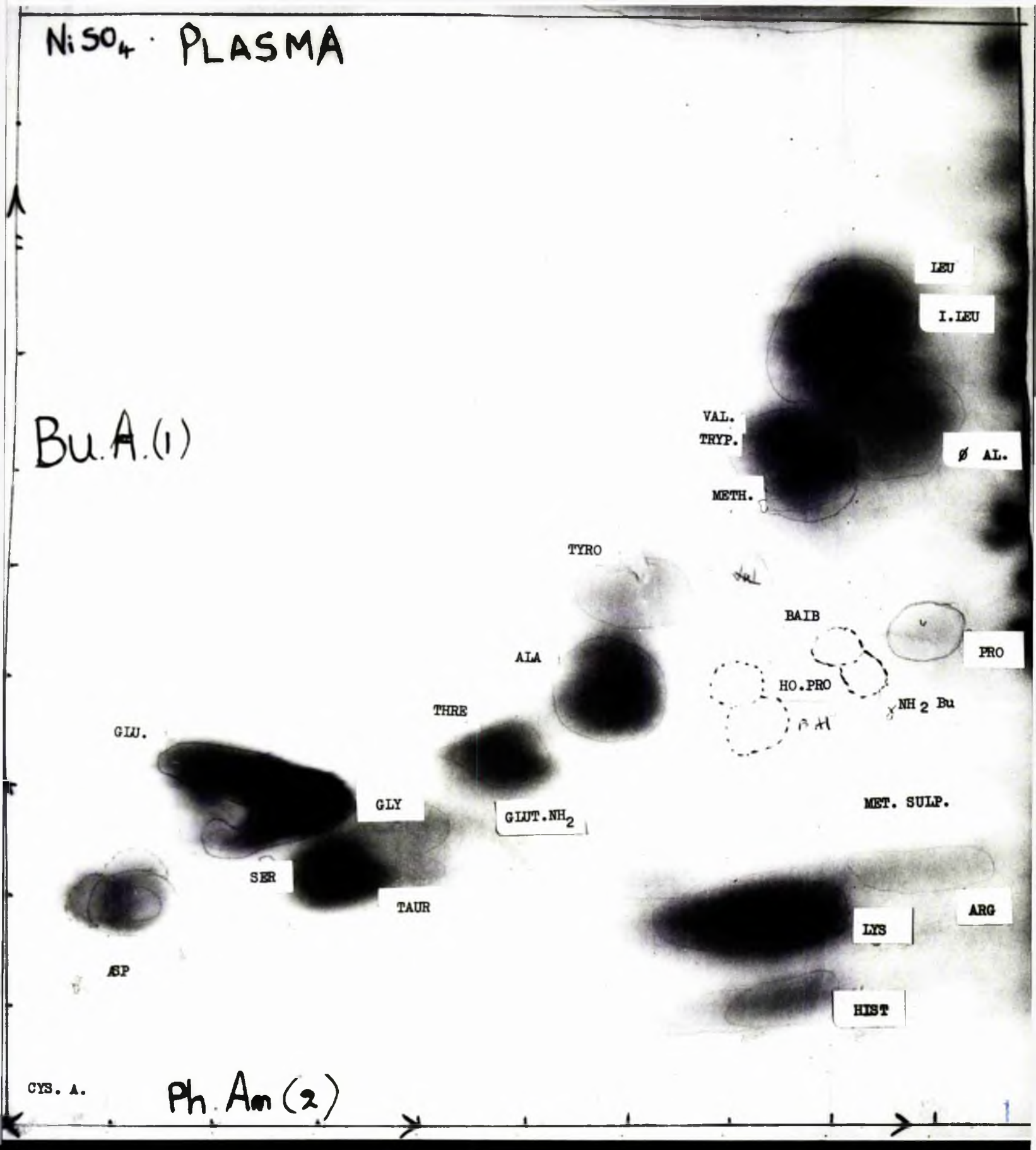


FIG. 10.43 Urinary Arginine



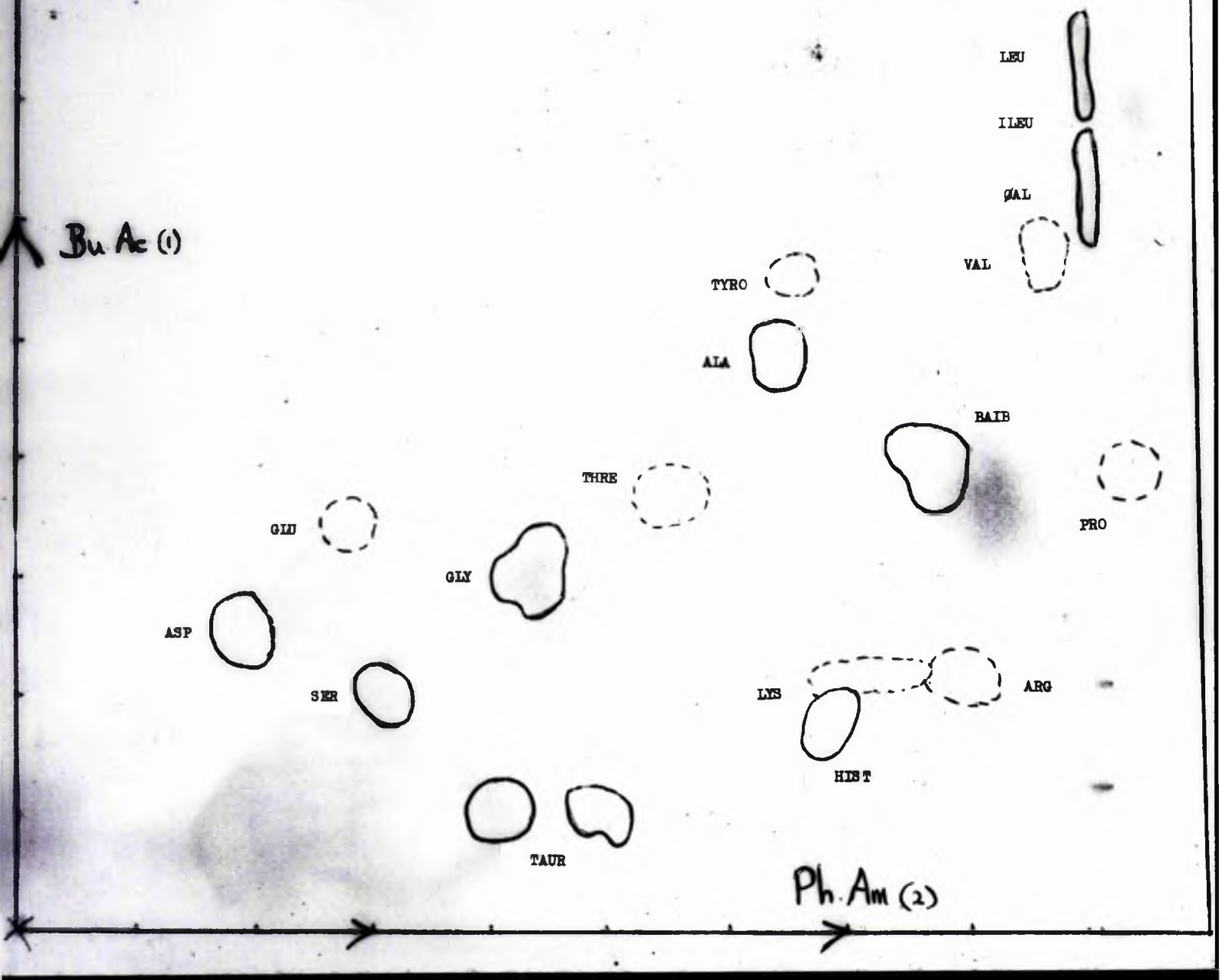

3
4
5
6
7



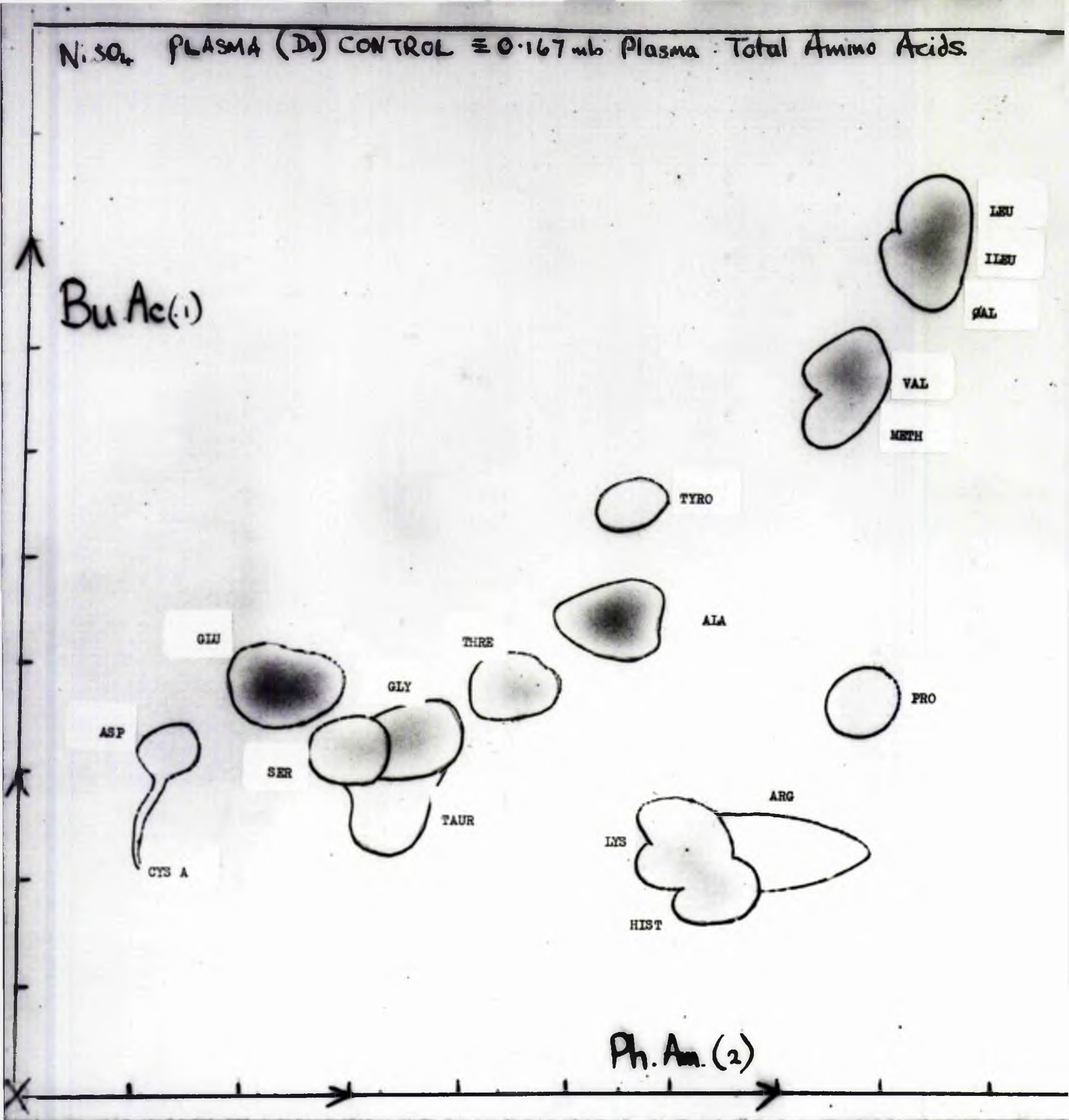
1. Plasma Amino Acid chromatogram (TOTAL) Control: 0.5 mls. Ninhydrin only: Nickel sulphate stain (N.S.S.).

5→2/1→1/0·1

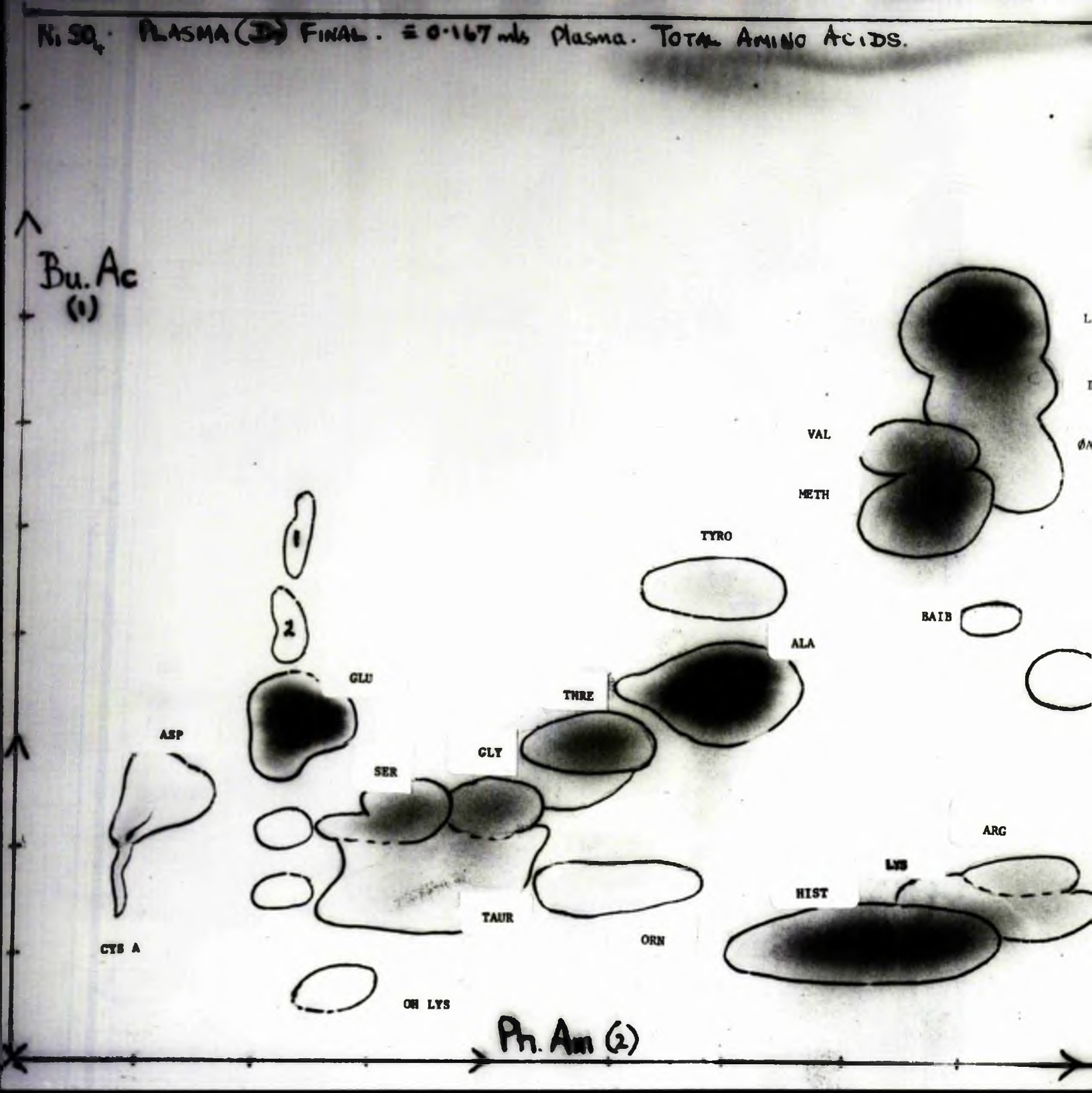
From Plasma Amino Acids : FREE : ≡ 0·25 mls Plasma (Nea)



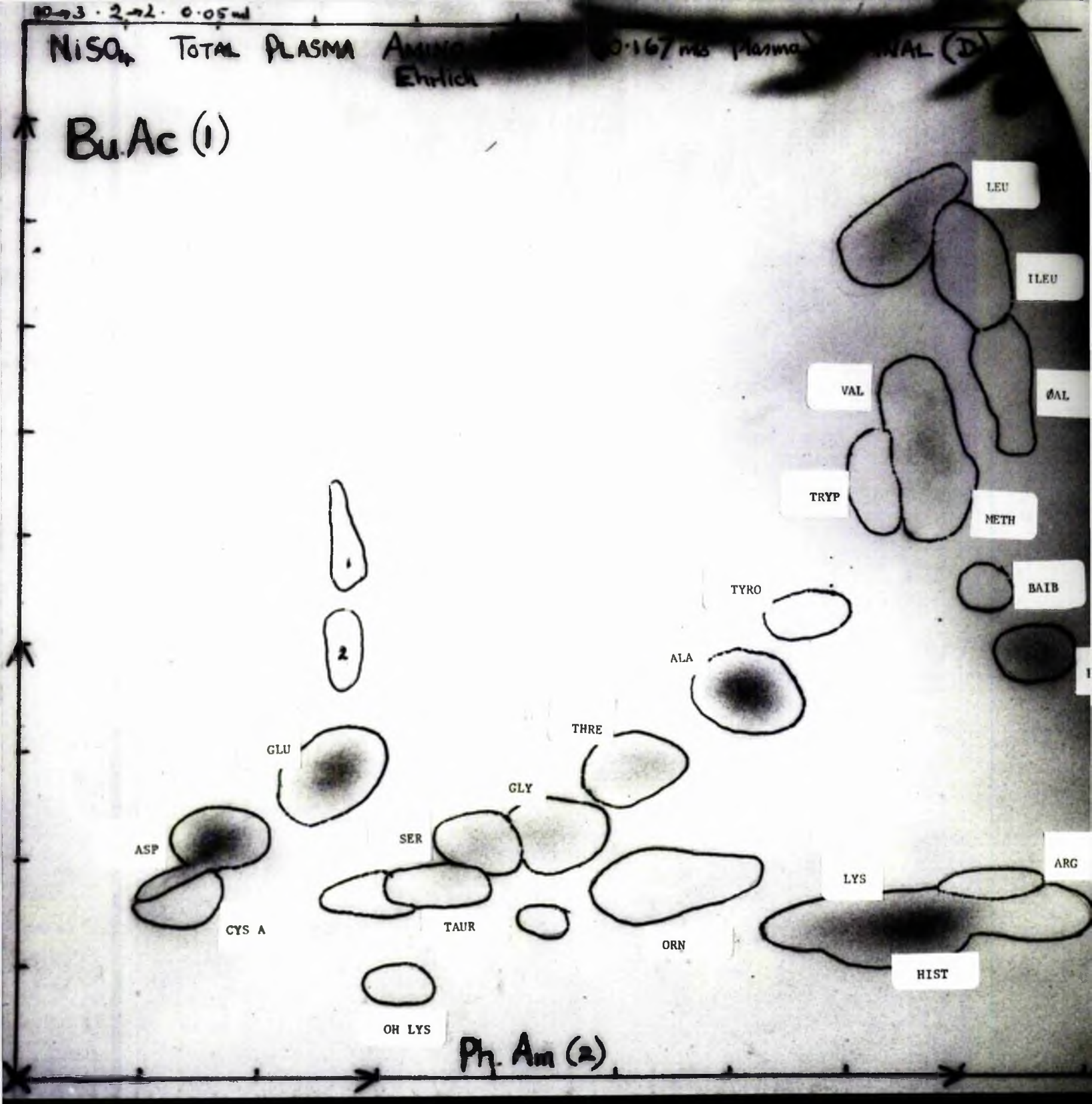
2. Plasma Amino Acid chromatogram (FREE only): "9 C.N". The above shows the increase in free plasma amino acids after a 2 hour perfusion. The control plasma (using the same volume, 0.25 mls) showed very few free amino acids: Ninhydrin only: N.S.S.



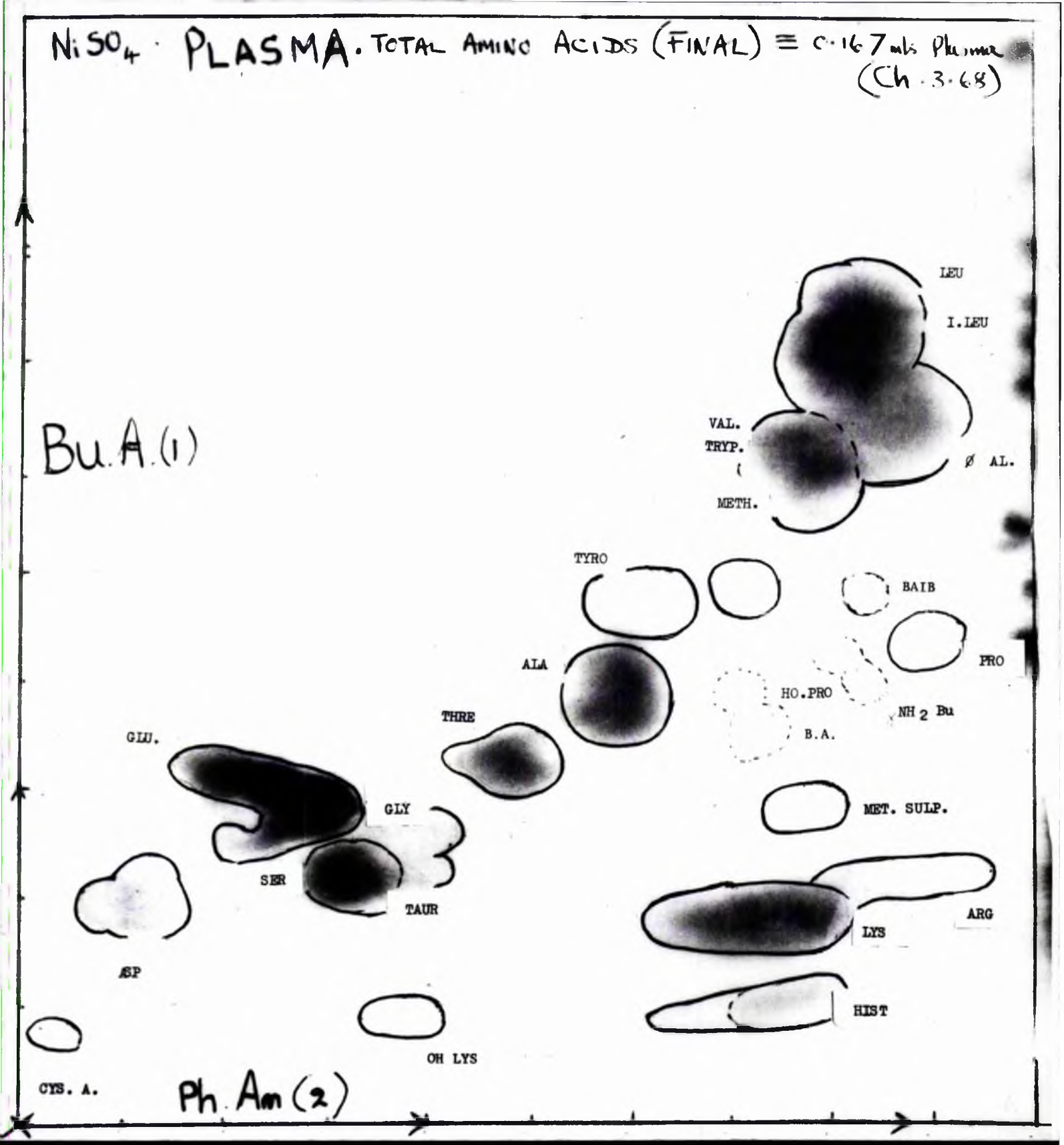
3. Plasma Amino Acid chromatogram (TOTAL) "13.R.D" 0.167 mls. Control : Ninhydrin only: N.S.S.



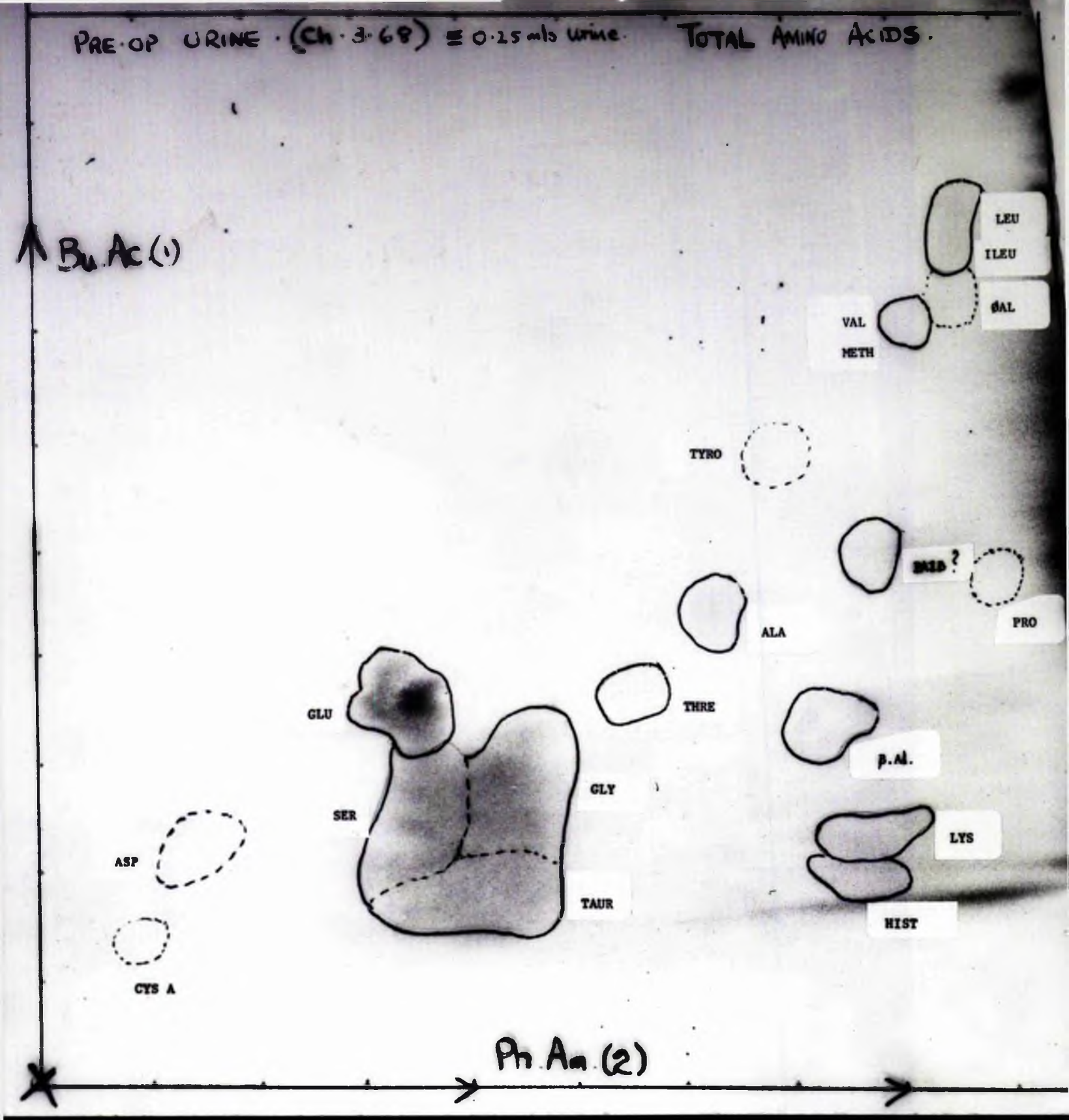
4. Plasma Amino Acid chromatogram (TOTAL) "13.R.D" : 0.167 mls. FINAL: Ninhydrin only. N.S.S. The contrast with the control is well marked. Spots 1 and 2 were unidentified but are NOT amino acids since 1) none have R_f values of approximately 50:25 and 40:23 respectively, and 2) no unidentified peaks appeared during the 'phoenix' analysis.



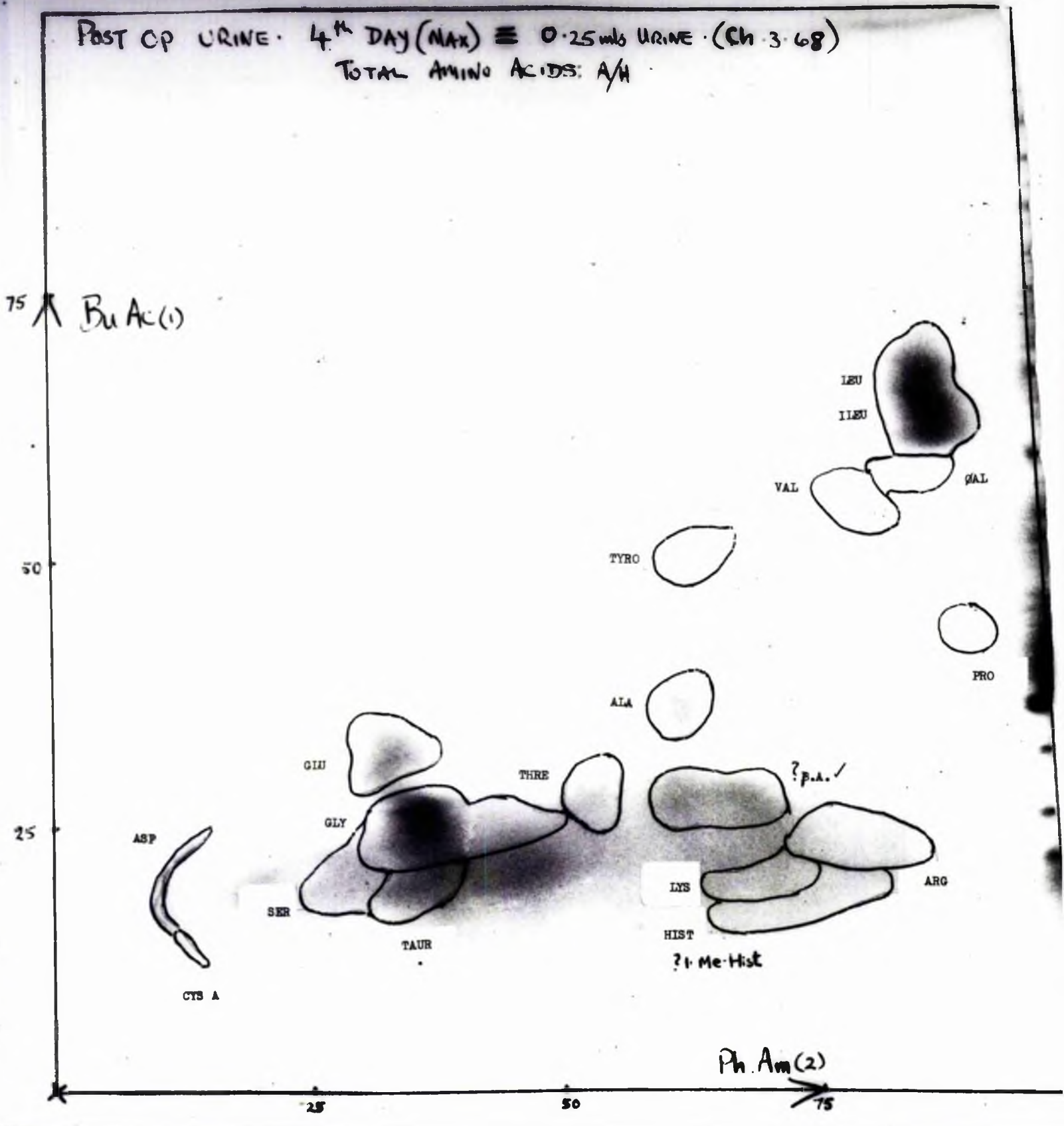
5. Plasma Amino Acid chromatogram (TOTAL) "13 R.D" 0.167 mls. FINAL. This is the same as '4' except that the Ninhydrin chromatogram has been stained using Ehrlichs Reagent followed by N.S.S.



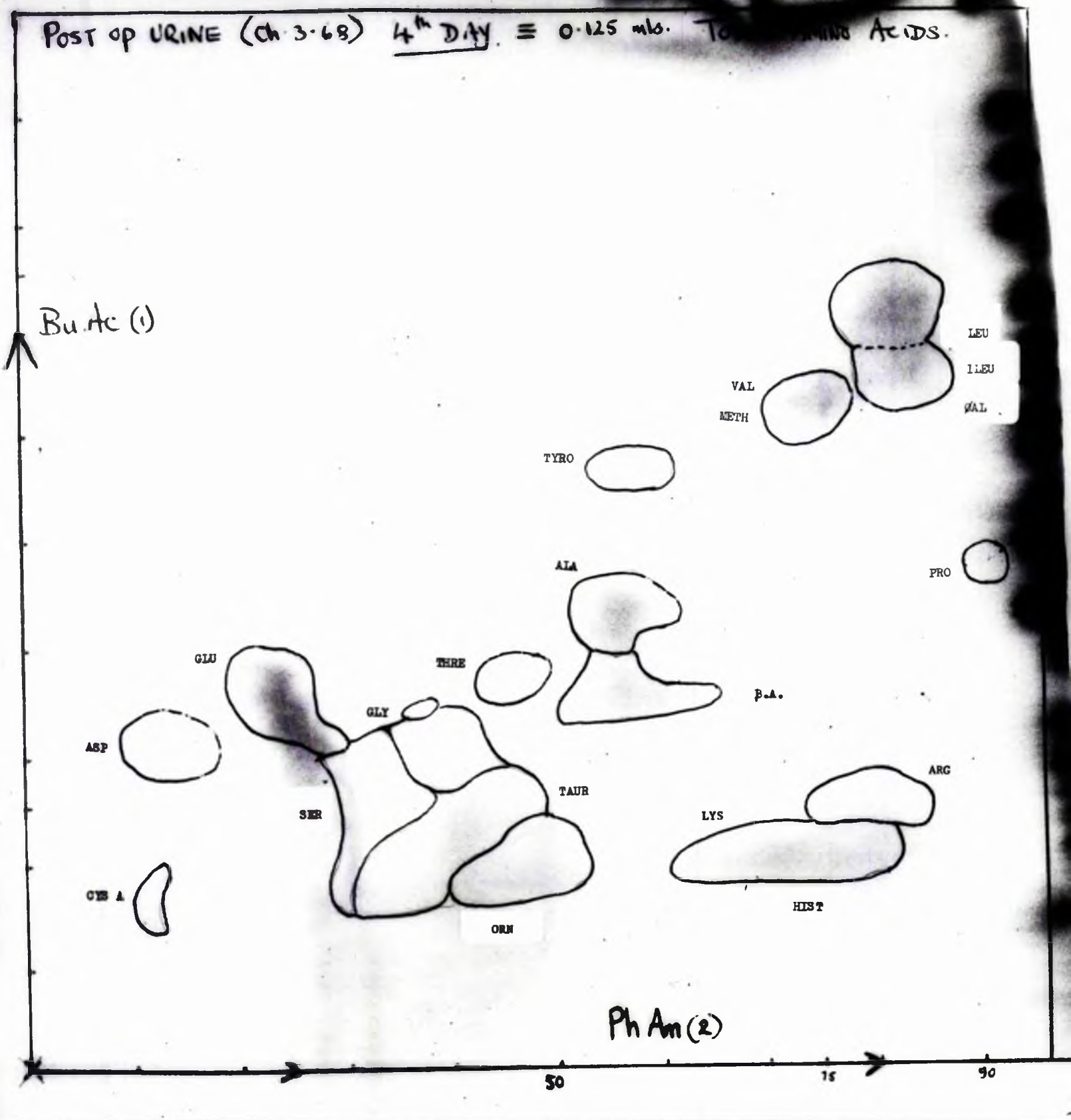
6. Plasma Amino acid chromatogram. (TOTAL)
"4 M.Ch" 0.167 mls. FINAL plasma. The similarity with '4' may be seen. Ninhydrin only: N.S.S.: The Control chromatogram was identical with '3'.



7. Pre operative urinary amino acid chromatogram (TOTAL) "4 M.Ch" 0.25 mls. Ninhydrin only: N.S.S.



8. Post operative amino acid chromatogram 4th DAY, (TOTAL) "4 M.Ch." 0.25 mls. Ninhydrin only N.S.S.



9. Post operative amino acid chromatogram 4th DAY, (TOTAL) "4 Ch". 0.125 mls: Ninhydrin only N.S.S.

HYPERAMINOACIDAEMIA AND HYPERAMINOACIDURIA

The hyperaminoacidaemia seen during perfusion has certain similarities and dissimilarities with changes seen in liver disease (Iber et al. 1957 and Nutr. Rev. Editorial, 1958, and burns Nardi, 1954).

The hyperaminoacidaemia of hepatic insufficiency concerns mainly methionine and tyrosine and to a lesser extent phenylalanine and threonine.

The urinary amino acid pattern seen in burns is similar to that seen in these cardiac perfusion cases and ordinary surgery, in that there is not only an increase in the non essential amino acids normally found in the urine but also an increase in essential amino acids not usually present. The latter were mainly.

Threonine	Isoleucine
Valine	Lysine
Leucine	Methionine

It is interesting that no increase in phenylalanine nor tryptophan occurred in burns. In perfusions a moderate increase in plasma phenylalanine is seen and a vast increase in urinary excretion of this amino acid. No changes were observed in plasma and urinary tryptophan however.

Therefore it is clear that the only difference between burns, and cardiac perfusions and ordinary major surgery is that in burns there appears to be no increase in plasma phenylalanine nor its urinary excretion whereas in the two types of surgical operations both plasma and urinary changes are seen.

Nardi has also shown that in less severely burned patients and minor surgical procedures, there is only a minimal increase in urinary amino acids - which were mainly non-essential.

Patients dying of burns show a massive amino aciduria of both essential and non essential acids and thus show very similar changes to those of major surgery "15 McC" and cardiac perfusions.

Chytil, 1956, has induced aminoaciduria due to toxic injury by injection of turpentine into animals.

Causes of the hyperaminoacidaemia

The exact causes of hyperaminoacidaemia cannot be stated at present but there are several practical possibilities for which the true answer can probably only be obtained by systematic radio-active labelling.

1) Haemolysis during perfusion increases considerably in an approximately linear manner. The plasma amino acid increase does not show this type of correlation, it being more sigmoidal in type. Human haemoglobin has a low isoleucine content 0-0 - 0.28% (Tristram and Smith)* and this

* Fig 10.49

amino acid showed very minimal changes during perfusion. Haemolysis therefore is a possible source.

2) Lipoproteins as well as haemoglobin are poor in isoleucine content (Shore et al. 1954) and to a lesser extent human plasma albumin and globulin, 1.47 and 2.23% respectively, (Tristram and Smith).* Thus degradation of plasma proteins (due partly to oxygenation during perfusion, proteolytic enzyme action, trauma, and partly to duration of operation) is a possible cause of some of these amino acid increases. Fibrinogen is relatively rich in isoleucine (4.8%).* It is well known that this protein (Mean 0.34 g/100 mls. Geigy) is increased 2-4 times, 4-7 days post operatively. This is just the time when the hyperamino aciduria is decreasing, thus signifying that the cause of amino acidaemia occurs early in the post operative or late operative periods, and is unrelated to denaturation of fibrinogen or its catabolism.

It is reasonable to suggest that if too great a protein degradation was taking place during perfusion than more direct evidence of shock would occur in the post operative period - which in fact is rarely seen. So far as albumin is concerned as a potential source of amino acids - Fleck and Munro, 1963, have shown that injury (? severity) has no effect on albumin catabolism. Davis et al. 1959,

* Figs 10·50, 51 and 52.

on the other hand, have shown that the I^{131} Albumin turnover is increased in burned patients.

Using C^{14} labelled amino acids and I^{131} labelled proteins McFarlane et al. 1960 have found that the albumin, globulin and fibrinogen turnover was not increased following fractures in rats or cortisone implantation. The specific activities of free amino acid carbon was increased 50% while the plasma amino acid concentration was decreased. This specific activity change may be due mainly to a change in the amino acid pool. Random fluctuations normally occur in this pool but these were greatly increased under the stress of fracture or cortisone administration.

Gillette, 1958 has reported an increase in α_2 globulins in goats suffering from shock.

Cuthbertson and Tompsett, 1935 have shown a decrease in albumin and an increase in fibrinogen and globulins (especially α_2 and β) in fractures, operations and burns in man.

Engel et al. 1943 and Russell et al. 1944a, studying the effects of haemorrhagic shock in rats, found increased blood levels of amino nitrogen due to increased catabolism of protein in peripheral tissues, and, presumably due to anoxia and decreased metabolic activity a decreased ability of the liver to metabolise plasma amino acids, (Engel et al. 1944

and Russel, 1944 b). Engel has implied that a decrease in deamination of amino acids occurs in shock. This compliments the findings of Van Slyke et al. 1944, who found that in Haemorrhagic shock in dogs the liver fails to remove amino acids from plasma confirmed by observation of decreased urea formation.

It is interesting however that Russel and Wilhelmi 1941 have demonstrated an improved rate of deamination in adrenalectomised rats during cortisone therapy, which may be due to improved tubular reabsorption.

The fact that in severe shock the liver may well contribute to an increase in plasma amino acids rather than increase their removal is suggested by Russell and Long, 1946.

Presumably the decreased ability of the liver to deaminate is a major defect in liver metabolism, since low blood urea concentrations are not accompanied by an increase in ammonia in the liver of shocked animals, Wilhelmi et al. 1945.

The inverse relationship that plasma amino nitrogen and elevated blood sugar levels have, is commented on in the section below on "Stress in Surgery".

Eades et al. 1953, has shown that peptides occur in the urine of injured patients.

In regard to the aetiology of decreased hepatic metabolism it is quite feasible that an increase of some plasma amino acids may act by feed-back inhibition and depress still further hepatic catabolic and detoxicating mechanisms.

It is also quite possible that during the operative and post operative recovery periods the liver not only has a decreased ability to remove plasma amino acids but may actually contribute to them.

If there is any degree of oliguria in the late operative and post operative periods this will increase plasma aminoacidaemia to even higher levels of toxicity and thus depress hepatic metabolism.

<u>The causes of the amino aciduria</u> may be

1) Simply an "overflow" mechanism due to plasma amino acid levels being in excess of the respective renal thresholds.

2) The depressive effect of the more toxic plasma amino acids on the renal tubular re-absorption mechanisms. One amino acid may block not only the tubular reabsorption mechanism of itself but also other amino acids (e.g. hereditary prolinuria). This is an uncommon condition in which

high plasma proline concentrations give rise to not only proline in the urine but also hydroxyproline and glycine.

Threonine and histidine excretion have been shown to be very susceptible to high concentrations of any other plasma amino acid by Kamin and Handler, 1951.

3) It is a remote possibility that any inherent inborn error of metabolism hitherto undetected will obviously greatly influence the exact amino aciduria found, especially as most of these "errors" involve more than one amino acid.

Dent and Schilling in 1949, have shown that rapid injection of proteins (gastro-enterostomy) in dogs gave large increases in most plasma amino acids in portal blood and this was associated with aminoaciduria. Although peak of hyperaminoacidaemia and hyperaminoaciduria occurs during low dietary intake in perfusion patients, the cause of the hyperaminoaciduria may have a common factor with the results of this experiment namely that it is due to simple amino acid overflow as mentioned in 1) above.

4) The increase of plasma amino acid concentrations following ACTH administration was suggested by Friedberg et al. 1947 and Li et al. 1949, and aminoaciduria due to the

same treatment by Ronzoni et al. 1953.

This effect of ACTH and adreno-cortical secretion upon nitrogen metabolites, amino acids and electrolytes is discussed in detail in the sub section below.

5) pH changes in the blood as a minor source of hyperamino-acidaemia during perfusion is a remote possibility. In work done by Walker et al. 1963 the mean pH range during perfusion was 7.5 - 7.4, and in the post operative phase 7.3 - 7.4. It is very unlikely that these minor changes would have profound effect on release of bound amino acids.

The affect of hyperaminoacidaemia on the osmotic pressure and buffering action of the blood is unknown.

It has been mentioned that up to perfusion "10 Mo" (inc.) hydrocortisone was given regularly during the operation. It is known that Lysine increases in both blood and urine during ACTH therapy and thus it is not surprising to find high concentrations of this amino acid in both plasma and urine in the first 10 perfusions when hydrocortisone was in fact given. In cases 11 to 17 however the plasma lysine values are less than 50% of the former values (1-10).

The urinary lysine changes between cases 1-10 and 11-17 are less dramatic mainly because (a) the plasma amino acid levels are still well above the renal threshold values and (b) There is a much wider variation of urinary amino

acid concentrations than there is normally with the plasma amino acids. This applies virtually to all amino acids.

Although especial reference has been made with lysine other amino acids are also increased under pituitary-adrenal stimulation, histidine and arginine are not.

Specific mention has already been made in the discussion of plasma and urine histidine changes during perfusion - of the probable significance if any of the histidine findings at perfusion to the histidine concentration in haemoglobin. Although free haemoglobin is liberated into the plasma during haemolysis, only severe destruction of the protein would liberate histidine in any significant quantities. Since histidine is only increased in moderate concentration in the plasma during perfusion it is very unlikely that such histidine has in fact come from this haemoglobin source, as there is such a vast discrepancy between the plasma histidine concentration and the histidine content of the total haemoglobin haemolysed during perfusion.

Secondly - whereas the rate of haemolysis follows an approximately linear course during perfusion, it seems that after an initial characteristic rise the plasma histidine concentration rises no more and assumes a "plateau" after 2 hours on perfusion. There is thus no linear relationship between these two criteria.

In the discussion of amino acid changes in both plasma and urine it is pertinent to stress that the amino acids increased most are not necessarily the most important nor the most metabolically active as compared with those few amino acids which appear to alter very little e.g. isoleucine, methionine and tryptophan. To illustrate this - the plasma and urinary alanine concentrations rose quite markedly during perfusion and as mentioned in the alanine discussion it is a non essential acid with little specific importance in general metabolism. Methionine on the other hand which alters quite markedly in the plasma as well, but at much lower concentrations, shows virtually no change from normal excretion in the urine during the post operative phase - the excretion following a "plateau", and as has been mentioned it is an essential amino acid with an important role in general metabolism.

The increase in plasma and blood sulfhydryl groups which occurs during perfusion may not only come from haemolysis and mild denaturation of proteins but also from active sulphur metabolism - through methionine, cysteine (cystine) glutathione, cystathionine and ergothionine.

The sulfhydryl content of urines would be a most interesting study to pursue in the light of the above knowledge. It would have to be done on fresh urines which due to logistics could not be done during this study.

It is worth mention at this point that one of the long perfusion patients "3 Ch" (3½ hours) had a stormy post operative period in that she was unconscious for the first 4 days post operatively, regained consciousness on the 4-5th day and relapsed back into unconsciousness for a further 3 days. There were no overt neurological signs during this period and not surprisingly pulmonary complications ensued at the end of the first week. Clinically then, there appeared no reason for her unconsciousness; - however amino acid and total nitrogen studies of the post operative urines throw some light of these facts.

95% of the plasma amino acids were considerably increased during this perfusion. Glutamic acid, leucine and lysine were raised to concentrations of 84.3, 61.3 and 52.2 mgms/100 mls. respectively by the end of the perfusion.

Post operatively large increases were seen in all the amino acids with the exception of methionine and possibly ornithine and hydroxylysine. Commonly there is a biphasic pattern with many of the acids, with maxima occurring around the 3rd and 6th days. The total nitrogens were done on all the post operative urines and this mimics these amino acid findings.

The patient regained consciousness on the 4-5th day post-operatively, and it was at this point that the lowest

total nitrogen figure was obtained for the whole of that week - 9,554 mgms/24 hours, see Fig. 10.44.

Normally 84% of the nitrogen in the urine is due to urea, 4.8% to total amino acids and 4.6% to ammonia.* It has been clearly shown that both amino acids and ammonia are increased during operations and post operatively, and it is thus clearly feasible that these substances and quite likely related metabolites are responsible totally or in part for the degree of toxicity and rate of post operative recovery.

No unidentified amino acids occurred in the plasma or urine as a result of perfusion. In contrast to Dimililer and Trout who found "an unidentified amino acid between proline and serine". Since no particulars are given (solvent systems) it is impossible to guess what this substance is.

URINARY TOTAL NITROGENS.

The post operative urinary total nitrogens of patients "4 M.Ch", "7 D.M.", "13 R.D.", "15 J McC", and "16 K.R. are seen in figs. 10.44 - 10.48. The results mimic the amino acid changes and the bactrian curve is well exemplified.

*Much of this ammonia is derived from glutamine.

FIG. 10·44 Total Urinary Nitrogen
"4. M.Ch."

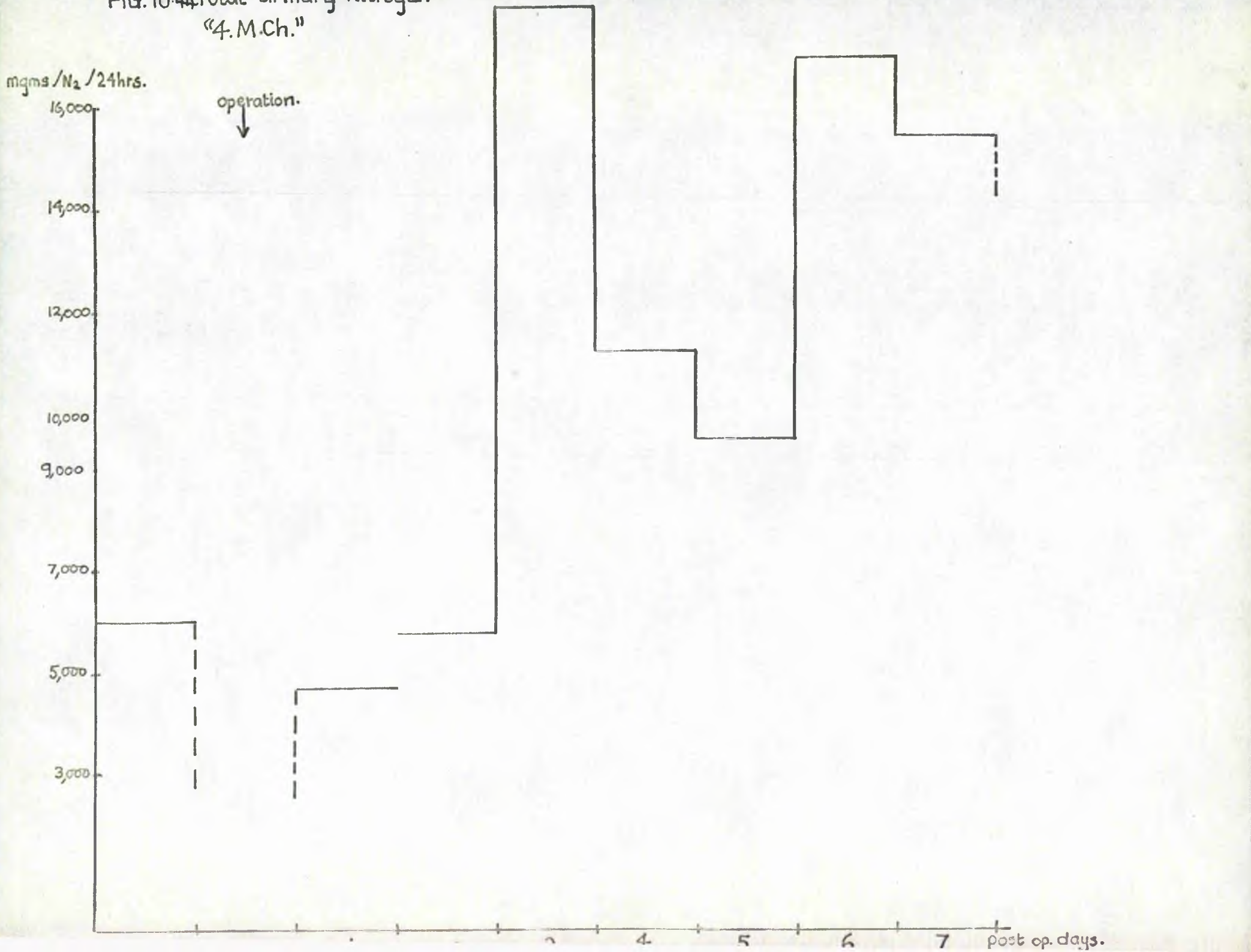


FIG. 10·45 Total Urinary Nitrogen "7. D.M."

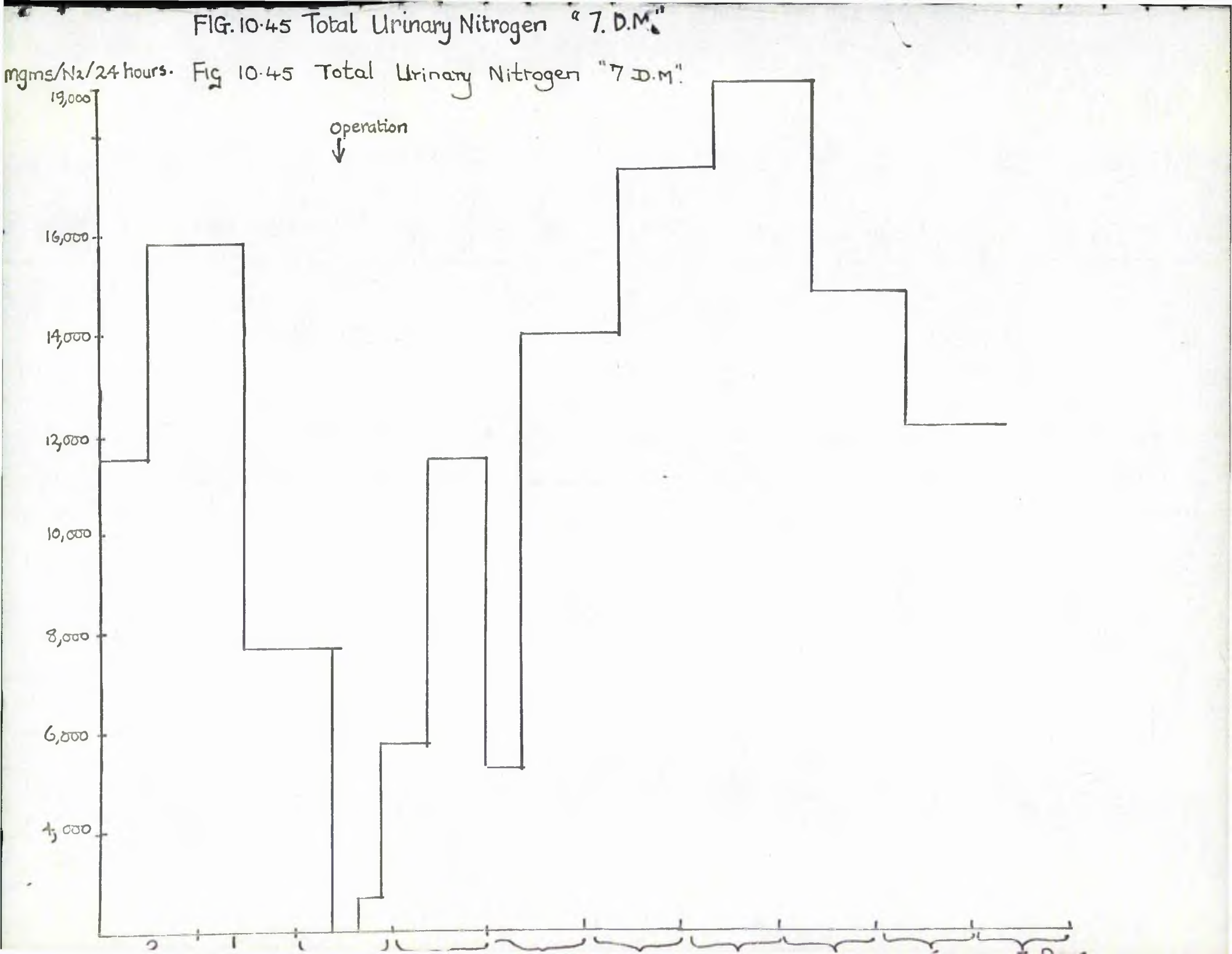


FIG. 10.46 Total Urinary Nitrogen. "13.R.D."

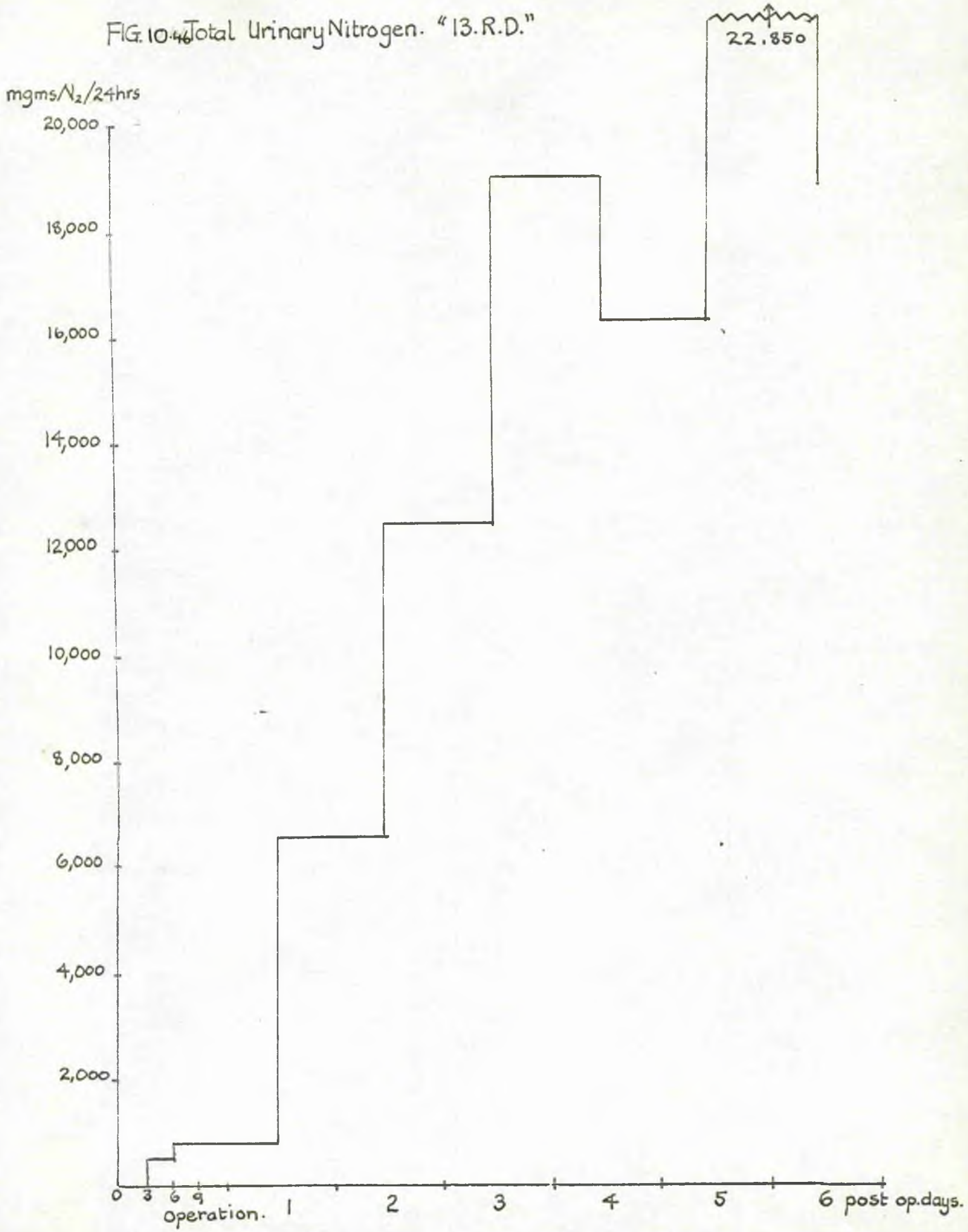
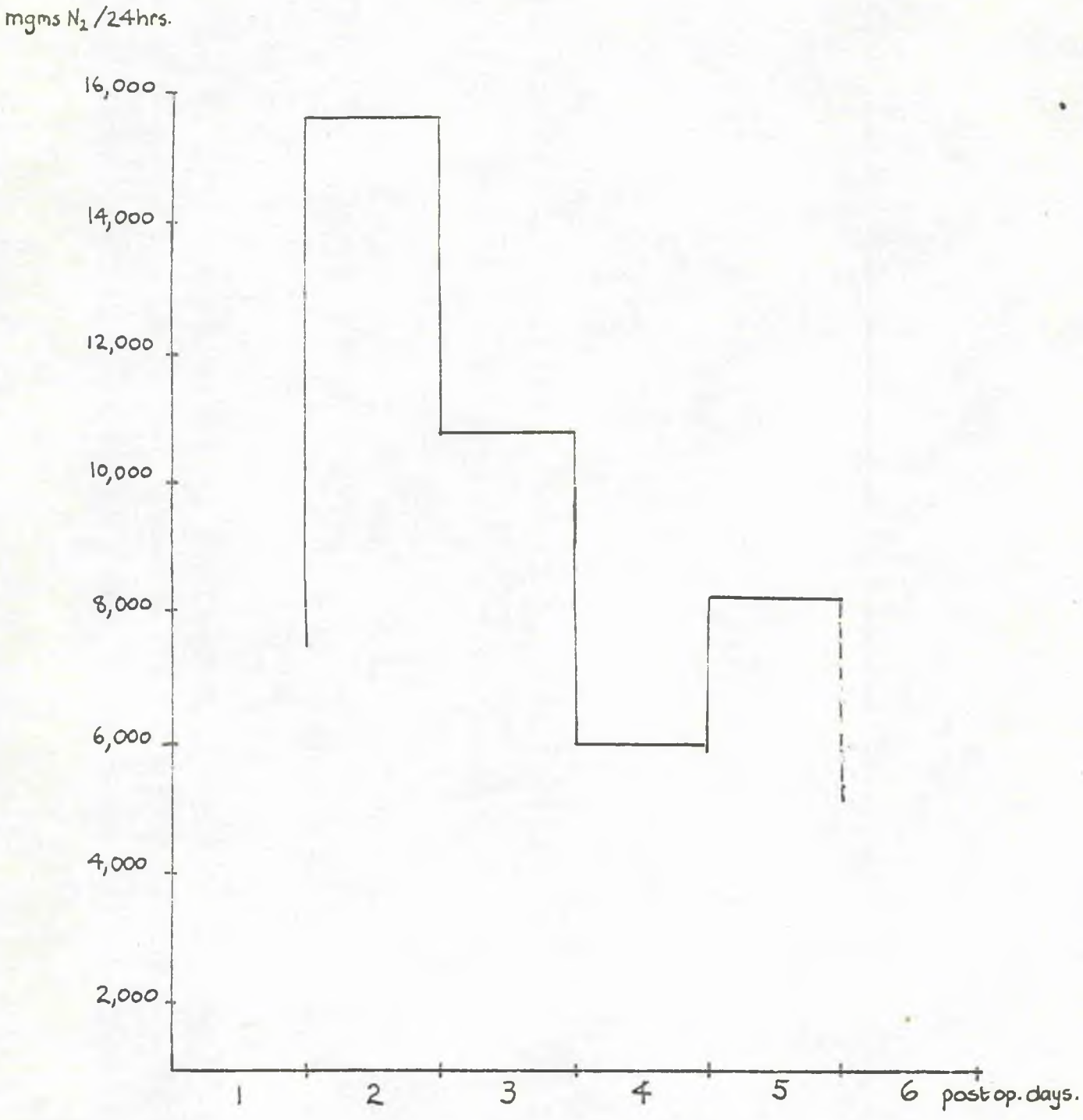


FIG. 10·47 Total Urinary Nitrogen. "15. J. McC."



Urinary Total Nitrogen "16K.R." 5 yrs.

Fig 10·48.

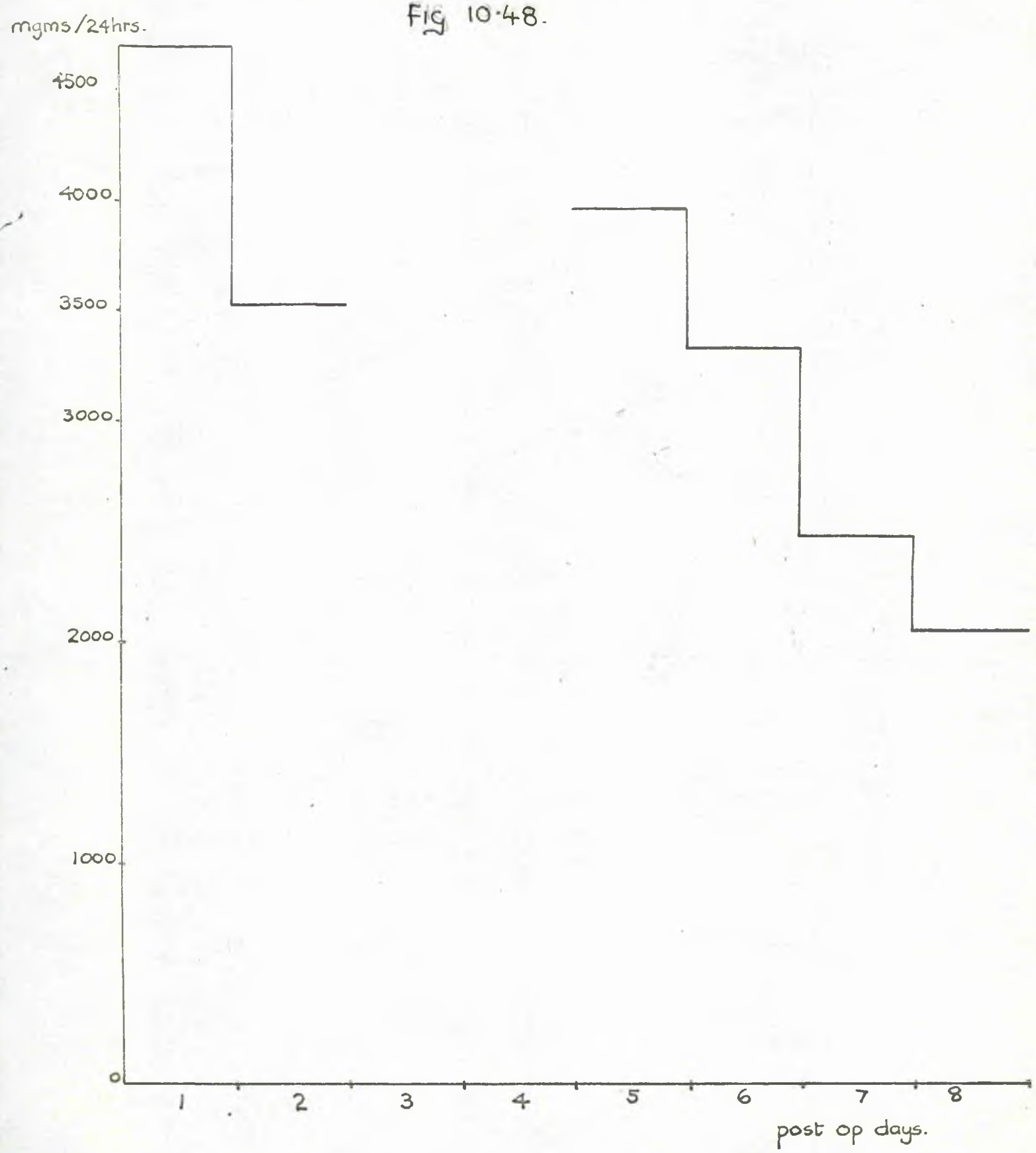


FIG. 10·49 Amino acid composition of Human Haemoglobin.
M.W. 64,500 : Total N. 16.9. (T. and S.)

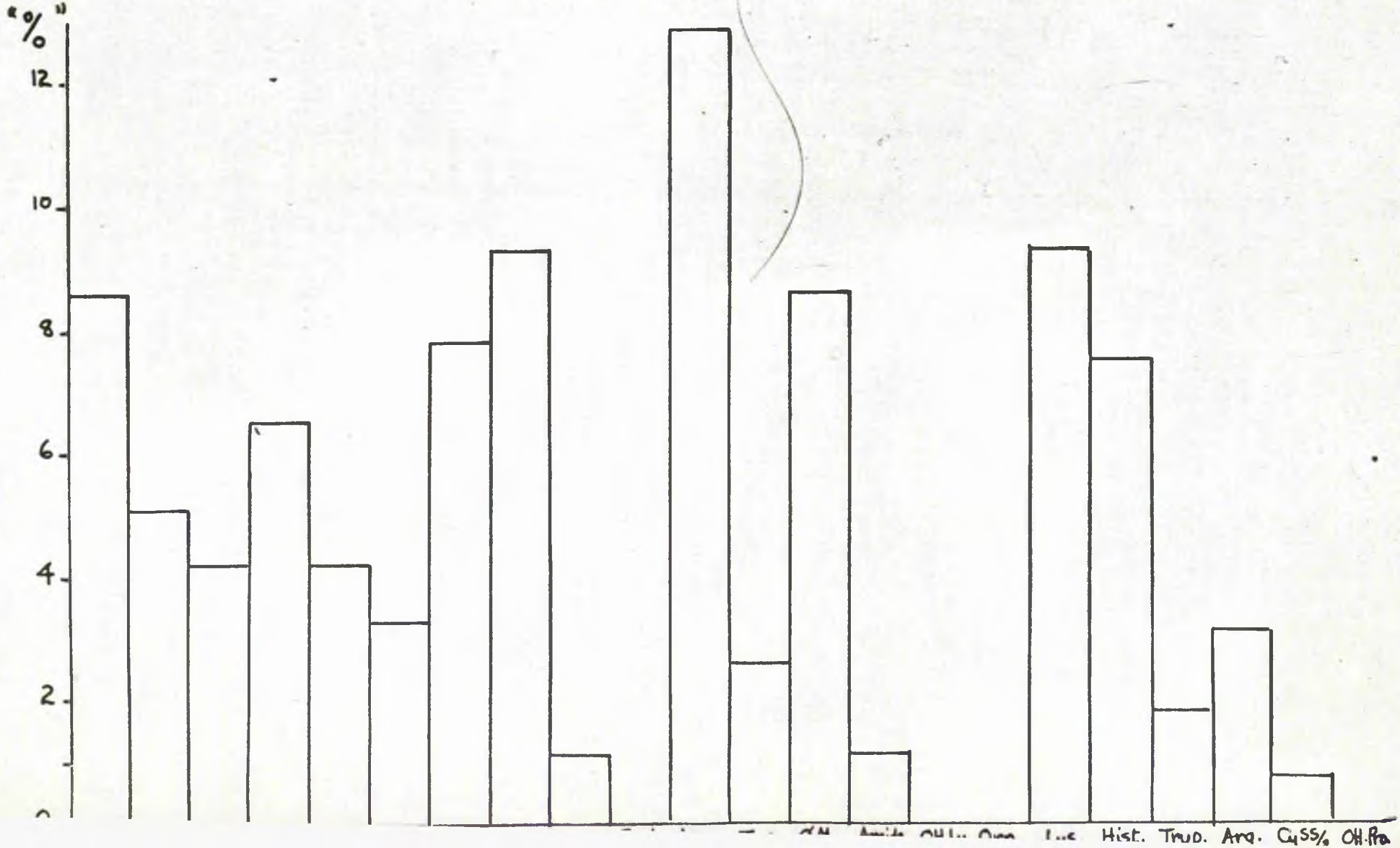


FIG. 10·50

The amino acid composition of Human Serum albumen

M.W. 68,000 Total N. n.d. (T. and S.)

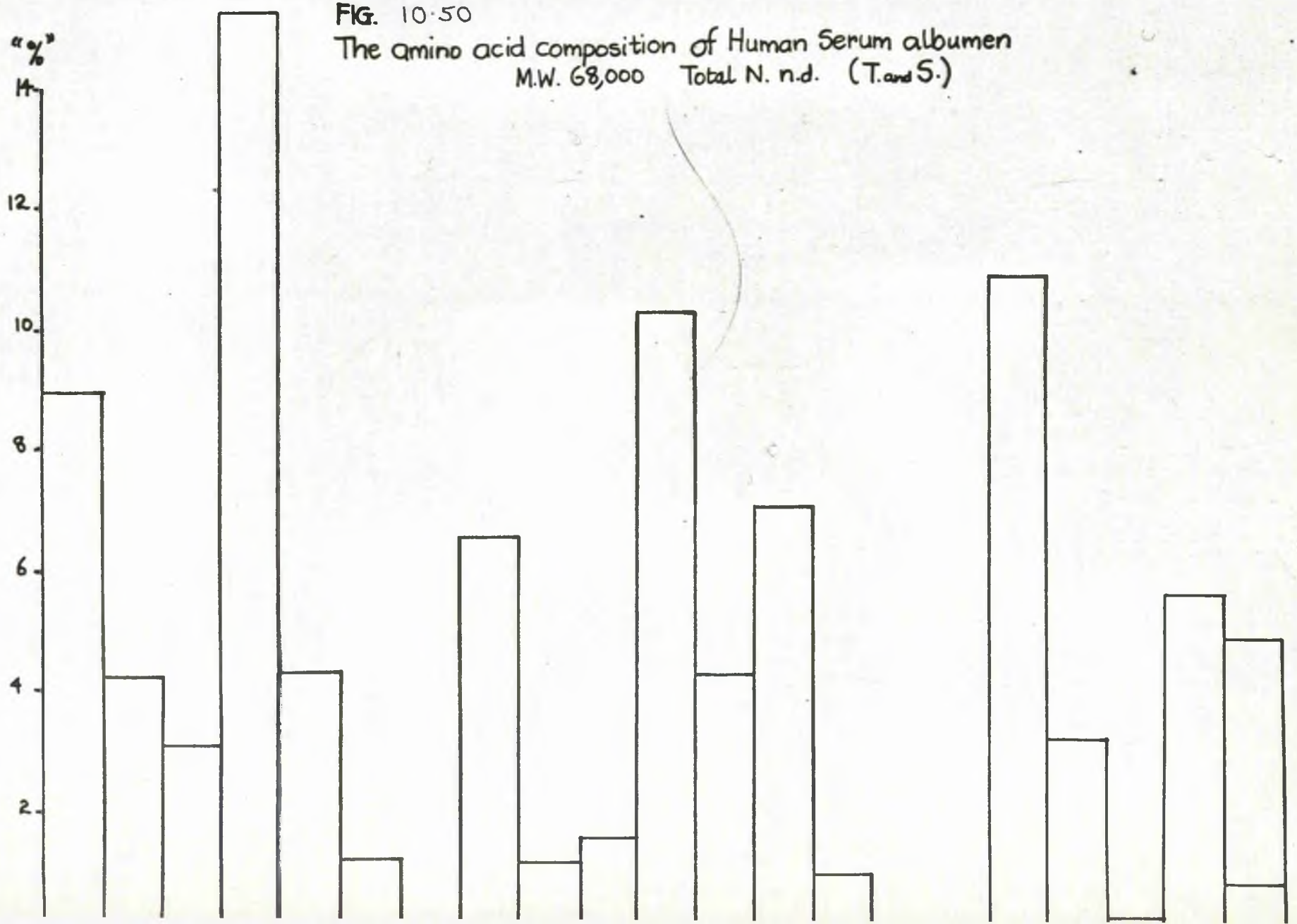


FIG. 10·51

The amino acid composition of Human γ globulin

M.W. 160,000 : Total N. n.d. (T. & S.)

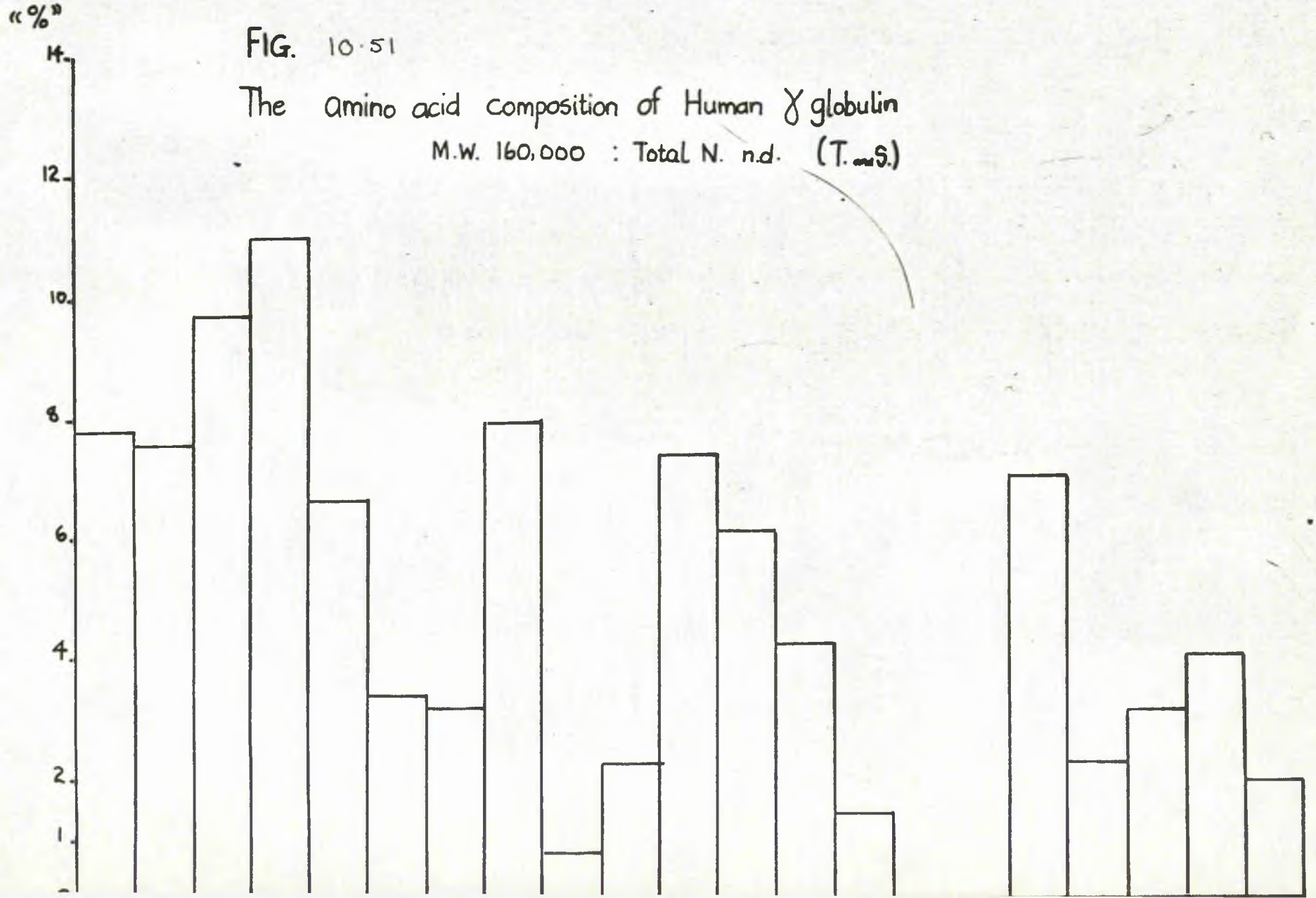
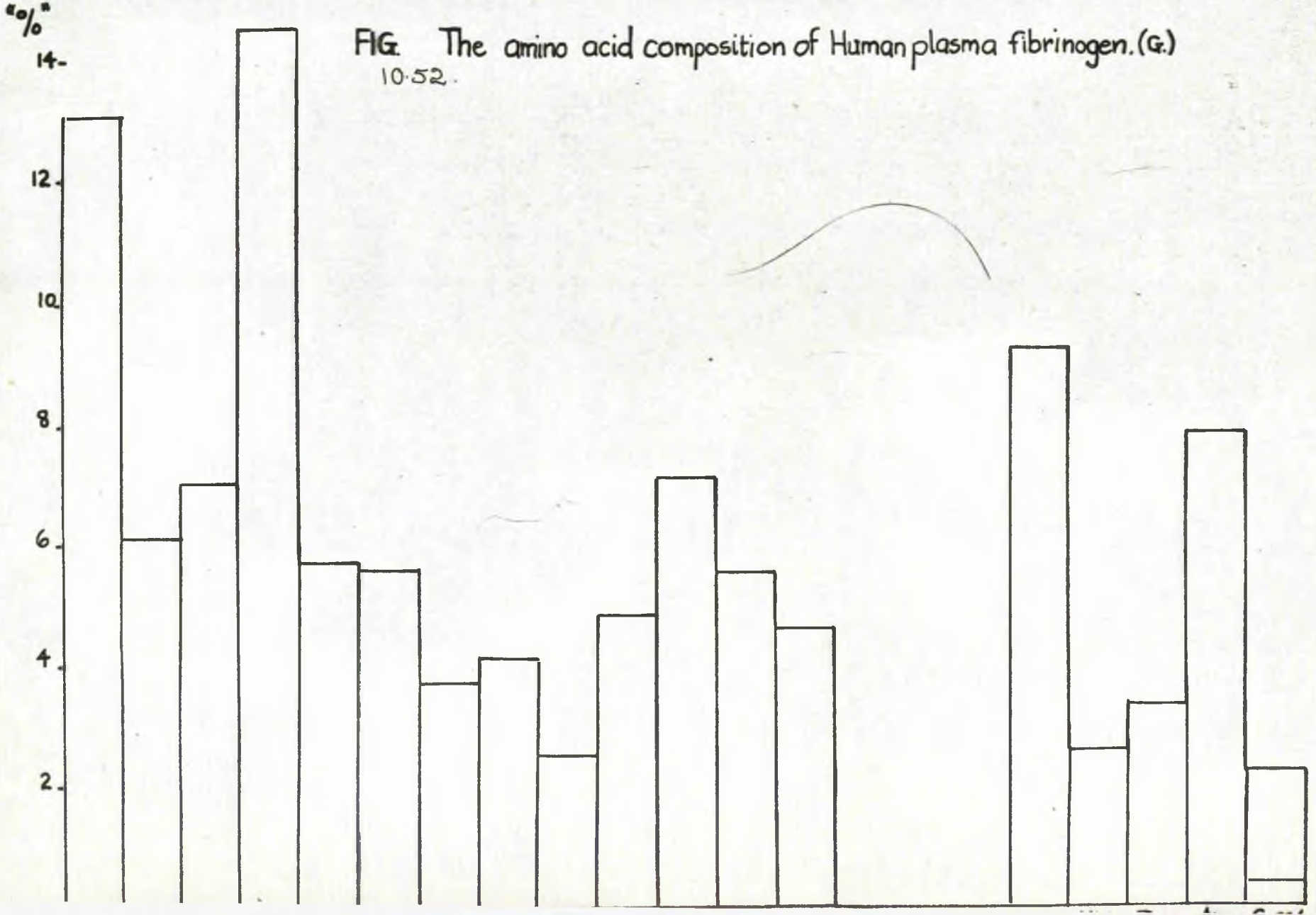


FIG. The amino acid composition of Human plasma fibrinogen. (G.)
10·52.



The effect of ACTH and adrenocortical secretion upon nitrogen metabolites, amino acids and electrolytes

Another very important factor concerning the amino acid pattern in surgery and afterwards is the reaction to stress and the effect of adreno cortical secretion upon the amino acid levels.

It has been shown that plasma and urinary ammonia and amino acids increase as a result of surgery. Other parameters for which values were sometimes obtained in these operations were the plasma and urinary urea, uric acid, and creatinine levels. Parameters not studied were purine bases other than uric acid, and creatine.

All these seven factors are increased in the urine due to stimulation by corticotrophin or hydrocortisone as a result of their metabolic activity.

Clark (1953) has shown that adrenalectomised animals given radioactive amino acids together with cortisol excrete more of the isotopic compounds in the urine than do control animals. Cortisol inhibits the amino acid incorporation into proteins.

A reliable criteria of adreno cortical function is the uric acid/creatinine ratio. After 25 mgm ACTH the ratio rises 62-130% (mean 91%). However the ratio is also increased to lesser values by fasting, (Fersham et al. 1948).

Although a number of creatinine and uric acid results were obtained they were not consecutive enough to warrant use of this ratio.

It is pertinent to mention again at this point that hydrocortisone was given to most of the perfusions up to and including "10 Mo", in concentrations of 100/mgms/hourly. It is not surprising therefore to find that in most of the long perfusions up to this case plasma amino acids reach as high as 80-90 mgms/100 mls. whereas in the perfusions after this case, maximum values are rarely above 30 mgms/ 100 mls. and these values are including three long operations of at least 3 hours, 2 perfusion cases and 1 control ("15 McC"). The results of 17-hydroxy corticosteroid estimations given in this section would support these findings in that :-

1) In the perfusion "10 Mo" when extrinsic hydrocortisone was given - plasma 17-O.H.C.S. levels were above 3000 µgms/100 mls. immediately after administration and did not fall below 1000 µgms within the first hour, whereas

2) in the remaining 3 cases in which extrinsic hydrocortisone was <u>not</u> given the 17-O.H.C.S. levels were around the physiological concentrations of 15-30 µgms/100 mls. plasma, indicating only a relatively minor "stress reaction" on the part of the adrenals during these operations.

In work done by Moore and Ball (1952) ACTH was administered to normal subjects in a moderate dose of 40 mgms/day. Nitrogen changes were virtually insignificant, and there was no change on cessation of the drug. Potassium loss (20-30 m.equivs/day) was not great and quickly reverted to positive balance upon cessation of the ACTH. Sodium conservation occurred during the therapy (as occurs during operations) and sodium diuresis appeared on ACTH cessation.

In a patient with chronic lymphatic leukaemia - who showed few signs of ill health - 100 mgms/day of ACTH was administered for 6 days at which time the dose was increased to 200 mgms/day. Negative nitrogen balance occurred and nitrogen excretion rose to a maximum of 21 gms/day upon the change from 100 - 200 gms. The negative balance which was 7-9 gms/day, did not return to normal until 2 weeks after the ACTH was stopped.

These changes mimic the findings in these perfusions and normal surgery in that urinary total nitrogens do not return to normal until the 8-10 th days after operation.

Potassium changes mimicked the nitrogen changes on the above ACTH therapy, and rapid urinary potassium loss ensued which increased when the ACTH dosage was doubled.

The converse changes take place with sodium. A slow but marked sodium retention occur on ACTH therapy, which

promptly changes to a diuresis upon ACTH withdrawal.

The urinary 17-ketosteroid response to prolonged ACTH therapy is not clear cut and is prolonged. 18 days of 200 mgms/day of ACTH was needed to produce an eventual output of 100 mgms of the ketosteroid.

The fact that a patient on 40 mgms ACTH daily showed no profound nitrogen change - even on cessation of the drug compliments the findings that

1) Nitrogen losses may occur in the absence of adrenal stimulation, and

2) Adrenal activity may take place in the absence of significant nitrogen excretion.

The importance of the above findings of Moore and Ball is that very similar changes occur during surgery and post operatively. The steroid secretion, nitrogen, potassium and sodium changes are all a function of the degree of stress imposed upon the body - be it surgery, fracture, starvation, burns or battle wounds.

Some differences do occur however between "in vitro" ACTH therapy and surgical response.

1) In "cold" ACTH therapy (i.e. normal patient) the negative nitrogen balance is slow to occur, whereas in surgery this nitrogen loss is rapid.

2) The potassium changes - which are zero or slightly

negative in ACTH therapy are quite marked in surgery. Reduction in urinary excretion usually occurring between the 3rd - 6th post operative day in normal health convalescence.

3) ACTH therapy is associated with weight gain, whereas in surgery weight loss is the rule.

4) During ACTH therapy the 17-ketosteroids excreted are far in excess of the changes seen in surgery.

5) The rate of sodium loss during ACTH withdrawal far exceeds the convalescent diuresis rate.

Moore and Ball in their excellent review have compared the above criteria in surgical patients undergoing different treatment.

Physiologically the severity and duration of a negative nitrogen balance may well have as one of its major reasons the provision of available protein precursors for wound healing. This involves.

1) The growth of fibroblasts.

2) The formation of collagen

3) The resumption of tensile strength

4) The regrowth of epithelium.

It is of interest that a rapid gain in wound tensile strength is normally coincident with a low level of 17-ketosteroid excretion in normal patients and a negative nitrogen balance.

The Stress of surgery

Various factors are involved which are responsible for producing stress as a result of operation. These are :-

1. Starvation
2. Immobilisation
3. Pain
4. Tissue injury
5. Inflammatory response and Infection
6. Anaesthesia
7. Apprehension
8. ? Oxygenation (perfusions) resulting in plasma protein denaturation.

Starvation is a short duration - 2-3 days and consequently of probably negligible importance in these perfusion results. If however it was prolonged, similar changes would take place to those seen in general surgery, except that instead of an initial sodium retention, a diuresis takes place for several days. Then the characteristic retention occurs as a result of adreno-cortical activation.

Immobilisation plays little part in nitrogen loss. Nitrogen, potassium, and sodium changes are minimal in control studies compared with those findings of surgery and trauma.

Pain - this is very important and may be lessened by promptly acting analgesics.

Tissue injury, inflammatory response (infection) are by far the most important causes of surgical stress. Discussed below.

Tissue injury due to surgery results in loss of protein due (a) to injury itself and (b) loss due to separation from the rest of the body. The inflammatory response is the converse in which initiation of repair processes requires protein participants. Associated with this, is a non specific general response to injury which leads to the negative nitrogen balance, loss of potassium, sulphur and protein. The balance pattern of these substances resemble each other. These post operative changes form "the post injury syndrome" in which both local inflammatory and general metabolic responses overlap.

The reaction of cellular components and haemostatic mechanisms will not be entered into here; suffice it to just mention them.

The post trauma period is associated with a hyperglycaemi probably due to adrenaline and partially to infused dextrose solutions (which is found in reduced concentrations in the adrenal medulla) after injury. Urinary excretion of both adrenaline and nor adrenaline is increased. However both ACTH and the adreno cortical hormones have this synergistic effect on blood sugar as well.

As a result of recent work it is possible that glucose may have a protective effect on the degree and rapidity of protein denaturation, (Personal Communication, Tristram, 1967). If this is so then presumably this transient hyperglycoaemia may be beneficial, and may be the reason for Green et al. 1949, finding that the plasma amino Nitrogen was inversely proportional to elevated blood sugar levels in battle casualties.

The release of potassium into blood after haemorrhage, muscle trauma, arterial occlusion or asphyxia may be an important factor in shock, Millican, 1960.

Cuthbertson 1942 and 1964, has shown that increases in nitrogen, phosphorus, sulphur, potassium and creatine occur in the urine following fractures and joint injuries - the maximum excretion occurring between the 4-8th days. The nitrogen increase was mainly due to urea excretion and the sulphur loss as inorganic sulphate as is usual. The excretion rate of both products is closely related.

The reaction to injury is a more active process than the catabolism due to disuse.

Modern anaesthesia is probably a minor cause of stress. Premedication has not only helped in this connection but has also reduced quite considerably pre operative apprehension.

Post operative apprehension is probably, when present, due to fear of pain and therefore attention must be given to as thorough alleviation of pain as possible.

It is difficult to assess the degree of stress that oxygenation of blood causes. Since denaturation of proteins appears minimal it is reasonable to suggest that it is a comparatively minor cause of surgical stress.

Of considerable importance is the cause of the fluid retention following trauma; for not only may there be some difficulty in the excretion of toxic metabolites but considerable electrolyte imbalance may occur if oliguria persists. It is possible that the effect of trauma sensitises the portal hypophyseal tract and thus provokes excessive ADH secretion. A secondary consequence of water retention is the inhibitory effect of increasing body fluid volume upon aldosterone production. This leads to increased renal salt loss and thus aggravates the hypotonicity of the body fluids.

This increase in body fluid could thus be partially responsible for the apparent decrease in plasma proteins seen occasionally in long perfusions (in the absence of additional extrinsic diluent e.g. dextrose saline).

Casey and Zimmerman, 1957, have shown that although high urinary aldosterone concentrations were found in the early post operative period of general surgery this was not maintained

during the initial period of positive sodium balance, and showed little correlation with the plasma sodium concentration. To date aldosterone may only act as an initiator in sodium retention following surgery.

Shu'ayb, Moran and Zimmerman, 1965 have shown that distension of the left atrium (by balloon) in dogs gives rise to a decrease in blood ADH and a 2-15 fold increase in urine flow. Release of left atrial tension as in mitral commissurotomy is followed by an increase in blood A.D.H. with consequent water retention.

Atrial stretch receptors also control the output of aldosterone. It is possible that the right atrium is partially responsible for sodium balance (same paper) and that the left atrium plays a considerably important role in water balance.

FINAL DISCUSSION

Sulfhydryl change on blood and plasma during perfusion and general surgery

Figs. 2A.10-2A.14 and 2B.12-2B.16.

The - SH content of both blood and plasma increases during operations to an extent which is roughly proportional to the duration. It is of importance to find that the results of the plasma changes mimic the whole blood changes, the only difference being a factor of 10^1 between the two, i.e. the plasma changes (0.05 mls.) range between 1.0 - 2.0 x 10^{-1} min/SH/L and blood changes (0.02) mls. between 3.0 - 4.0 (x 10^1) mM/SH/L. Bearing in mind the difference in volume between the plasma and blood used in the assays it is obvious that there is approximately 10^2 more - SH in the blood than in the plasma - this, as has been shown, is largely due to the - SH content of the Haemoglobin in the blood.

If one assumes (for the following discussion), that there is a mean Hb percentage during perfusion of 80% and that the m.W. of Hb is 68,000 then the Hb molarity is approximately 1.765 x 10^{-3} M (using 15 gms Hb/100 mls. as 100%) in the blood. Since 2 -> 6 "molecules" of - SH are

available in the Hb molecule* - according to the state of denaturation - it is clear that the majority of SH groups present in whole blood are due to haemoglobin.

In native haemoglobin the - SH content is approximately 0.09705%

and in denatured " " " 0.291%

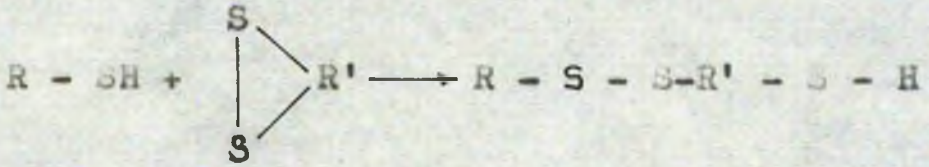
It has been shown that total haemolysis of blood - by water leads to a large increase in detectable - SH groups (Fig. 2A.9) 1.25 6.72×10^{-1} mM/SH/L. That this was due to largely haemoglobin and not the plasma proteins was suggested in a preliminary experiment in which plasma was removed prior to haemolysis of the red cells. The results were identical.

It is pertinent to mention that significant denaturation of Haemoglobin does not appear to occur during open cardiac surgery since it does not lose colour during this period and there is also no evidence that the O_2 carrying capacity is reduced.

It is seen in the Melrose experiment (M.E.) that there is a very large increase in detectable - SH groups during oxygeneration and that haemolysis increases proportionately. The latter was not estimated by the cyanomethaemoglobin method but by related - SH assay a good approximation of haemolysis during "the Melrose experiment" was found.

*Ingram states 4 - 8 -SH groups.

It is strange however that in the - SH assay of blood (M.E.) by the D.T.N.B. method the changes are not correlated with the - SH plasma changes (NEM) after 6 hours oxygenation. This is difficult to explain, however there is one possibility - whereas N. Ethylmaleimide detects only the more easily accessible and free - SH groups present, the extra - SH groups which DTNB is capable of detecting are more readily oxidised, or destroyed in these last four hours in the Melrose closed circuit. One has still to explain the fall at 6 hours (otherwise one could possibly expect a plateau between 6-9 hours) - this may be due to a chain reaction - similar to that mentioned in denaturation, Section 2, i.e.



in which an intramolecular disulphide linkage is replaced by an intermolecular linkage, thus the new - SH group is free to react again with another disulphide. If this process was taking place it would indicate an irreversible denaturation state which would indeed explain the whole blood changes after 6 hours of oxygenation.

Although albumin too has a number of sulfhydryl groups and disulphide linkages, it would appear from the

electrophoresis results that little change in the plasma proteins per se takes place up to 3-4 hours of perfusion.

In one of the specimens of blood taken from "15 McC" - oesophageal cancer patient - the plasma was accidentally grossly haemolysed and not surprisingly a very large increase in - SH content was found. This is obviously not recorded (30 min. aliquot) in Figs. 2A.10 and 2B.15) but it does reinforce the fact that increased - SH content is seen with increased haemolysis.

Since blood on being haemolysed is contributing free haemoglobin (and thus - SH groups) to plasma, it could be expected that plasma - SH assay would show a greater increase than blood - SH, with duration of perfusion. This is not in evidence probably because of the relatively small concentration of these groups in both fluids in the individual assays.

The rate of - SH increase in blood and plasma does not appear constant - mainly due to small blood or diluent additions during surgery. The results seen in the figures are the mean values of at least two and usually three assays.

The great variation seen in the first operation "I.J.M." is due to the large volume of added blood (12 litres) and diluent during the 4 hour perfusion.

The values of children on perfusion and the control general surgical patients are higher than the adult perfusion

patients because less diluent was needed, if at all, in the former patients, especially the children where blood is used rather than glucose.

The initial -SH values found during perfusion in both plasma and blood are slightly lower than normal controls simply due to this small degree of dilution.

It is possible that increase in small sulfhydryl containing molecules (glutathione, cystathionine, ergothionine, and sulphur amino acids) may contribute in some part to the overall sulfhydryl increase. It must be borne in mind that glutathione is liberated into the plasma upon haemolysis and that the sulphur amino acids are increased during perfusion.

The in vitro experiment of prolonged bubbling of oxygen and nitrogen through a small quantity of blood gave interesting though somewhat equivocal results. Oxygenation produced a decrease in detectable -SH groups using both NEM and DTNB for detection. This may be due to the small volume of blood used thus causing premature oxidation and destruction of the groups.

Using NEM as -SH agent during the nitrogenation, it is quite clear that the gas prevents this active -SH loss so apparent in the oxygenation experiment.

The Melrose experiment however does show conclusively that oxygenation does increase blood -SH.

As regards the participation of plasma proteins themselves as a possible source of - SH groups the following facts are of interest.

In the DTNB section it is seen that strong molar urea concentration failed to show any increase in - SH content of plasma, Fig. 2B.5. This agrees with Ellmans results. This therefore suggests that in spite of a number of - SH groups being present in the plasma proteins (especially albumin) they are not easily accessible to this denaturing agent. It therefore suggests that oxygenation as a minor cause of denaturation may equally well have little success in liberating these groups.

The other fact worthy of mention is that acetone appears to convert Hb Fe^{++} (ferrous haem) to the Hb Fe^{+++} (ferric), and that it also oxidises - SH to - S - S -, but since acetone increases - SH content in blood, these facts appear contradictory. The ferric haem however may use the globin portion of haemoglobin or -S-S- links in other erythrocyte proteins as a source of disulphide for reduction to -SH.

SULFHYDRYL REAGENTS

As regards choice of sulfhydryl reagents N- ethyl maleimide (NEM) was found to give accurate consistent results with plasma but could have been used with equal success with blood.

DTNB gave consistent results using whole blood, and was preferable to PNPD because of its solubility in aqueous solution. PCMB gave concise information on the nature and reactivity of - SH in the Hb molecule. All three reagents were used not only to complement each other but to also demonstrate differences in the quantitative estimation of -SH groups.

PCMB could have been used for the routine assays but the respective titrations of blood aliquots were found to be time consuming and thus put the stability of the - SH groups, to be assayed, at risk. The reagent was used however in the assay of control and final blood samples from the first operation "I. J.M.", but due to the significant haemodilution during the four hour procedure, the final results were only barely increased over the control as shown in the more rapid estimation by NEM and DTNB, Figs. 2A.10 and 2B.12.

As has been mentioned P.N.P.D. is the water soluble derivative of D.T.N.B. Since the latter was used initially with good results it would have been superfluous to use P.N.P.D.

The other methods mentioned were all investigated initially, but either due to absence of apparatus or superfluous nature of use they were not used in the routine analysis of samples.

Blood denaturation in open cardiac surgery

It is clear that any denaturation occurring in blood as a result of oxygenation during perfusion is of small magnitude for the following reasons.

1). The increase in - SH groups is largely accounted for by the degree of haemolysis. Therefore the plasma proteins contribution is small.

2). The results of electrophoresis and preliminary column chromatographic procedures show that only minimal protein changes take place as judged by intensity of bands and migration position compared with control. The difficulty in elucidation of the β and some γ zones due to similar migration of the haemoglobin (free) has been commented on and it is reasonable to assume that since albumin, α_1, α_2 and late γ components are not altered, then it is unlikely to see detectable changes in the β and early γ migrations.

3). Although turbidity and viscosity of plasma have been shown to be increased it must be borne in mind that other factors do influence these changes :-

a) Recently as mentioned in the turbidity section - cephalic stimulation of cats and dogs under stress results in increased plasma turbidity, the cause of which is unknown.

It is difficult to assess the degree of cephalic stimulation in humans.

b) The addition of blood during perfusion from donors in the post prondial state will increase plasma turbidity. Viscosity will also increase if this blood addition follows dextrose saline administration.

In the majority of perfusions minimal extra blood or diluent was added to the circulation after full perfusion had commenced. If this was found to be necessary it was added slowly over 20-50 minutes thus affecting the overall quantitative changes to only a very small degree.

c) Haemoconcentration increases turbidity and viscosity. Serial sampling during perfusions of haemoglobin, p.v.c., and electrolytes show that normal blood homeostasis was maintained and successive perfusions gave greater accuracy in maintenance of blood (and body) chemistry.

4). Ultracentrifuge studies show that no cleavage of large (protein) molecules occurs during perfusion.

5). If significant protein denaturation took place during perfusion it would not be surprising to encounter shock and/or renal failure post operatively. This in fact is rarely seen.

The renal changes that are suggested however are undoubtedly due to the large plasma amino acid increases.

The adrenal cortical response and amino acid changes

Advantage was taken during this perfusion study of relating the adrenal cortical response with the amino acid changes. It has been shown that during extrinsic supplementary hydrocortisone therapy of 100 mgms hourly the maximum plasma amino acid levels reach 100 mgms/100 mls, whereas in perfusions in which the hydrocortisone was witheld the maximum amino acid levels reached was only 30-50 mgm/100 mls. plasma.

Thus the mean plasma amino acid concentration is reduced some 50% in the absence of additional hydrocortisone.

This is an important result from this work and strongly suggests that more stringent control must be exercised in the use of hydrocortisone in cardiac surgery.

The results of the Melrose experiment (see Appendix 2.xii) indicate that despite a marked increase in haemolysis, 5% in 9 hours there was only a minimal increase in amino acid levels of 5-10%. This surely suggests that there is no relationship between the degree of haemolysis and the increase in blood amino acids which takes place in perfusion.

Another main cause of the rise in amino acids in these patients is probably due to a reduction in hepatic function which is usually present. The same rise in amino acids is seen in general surgical patients where depression of hepatic blood flow and function is known to occur. These facts suggest that the amino acid changes are due to general

surgical trauma and not to any specific factor of cardiac surgery.

The plasma amino acid increase is related approximately to the duration of operation.

There was no significant difference between the plasma values found at perfusions and those in general surgery.

The amino acids most increased were aspartic and glutamic acids, leucine and lysine. Of these lysine only is one of the known most toxic amino acids, (the others being histidine, tryptophan and tyrosine). All the other amino acids were increased to values approximately in the 10-30 mgms/100 mls. range, with the exception of methionine, hydroxylysine, ornithine, isoleucine and usually cysteine whose values remained very low. Tryptophan was present in just detectable concentrations.

The difference in urinary excretion of amino acids with and without hormone therapy is much less clear this is undoubtedly due to the several factors affecting renal function e.g.

1) the concentration of plasma amino acids
2) the renal threshold of the individual amino acids
3) the toxicity of the " " " "
4) the alteration of excretion of one or more acids due to an increased concentration of certain other amino acids.

The appearance and concentration of urinary amino acids is thus a resultant of many varying factors the most important being the appearance of urinary amino acids due to simple "overflow" and due to a toxic effect on the renal system.

The post operative urinary amino acids were all greatly increased in both cardiac and non cardiac operations with the exception of only ornithine, hydroxylysine and ? methionine. In regard to methionine low values were recorded in the perfusion cases but a large increase was seen in the control operation. Although no cysteine values were able to be obtained in this control operation, all large increases are observed in the perfusion cases. The importance of the sulphur amino acids has been discussed and it is not unlikely that they may contribute in small part (only) to the increase in blood - pH. However it is important to stress the low levels of plasma methionine and usually cysteine which were constantly found in all operations, which however is not necessarily synonymous with having little metabolic activity or importance.

Although, as mentioned above, the majority of urinary amino acids were increased, glycine, alanine, valine, isoleucine, leucine, tyrosine and phenylalanine gave appreciably lower concentrations in the non cardiac operation (15)

although this was of such long duration (3 hours). However it must be mentioned that there is a wide variation in urinary metabolites, and more post operative urinary amino acid studies on cardiac patients must be done to show the standard deviation of individual acids.

It would appear from this urinary amino acid picture that there is greater catabolism in post perfusion patients than in patients after general surgery.

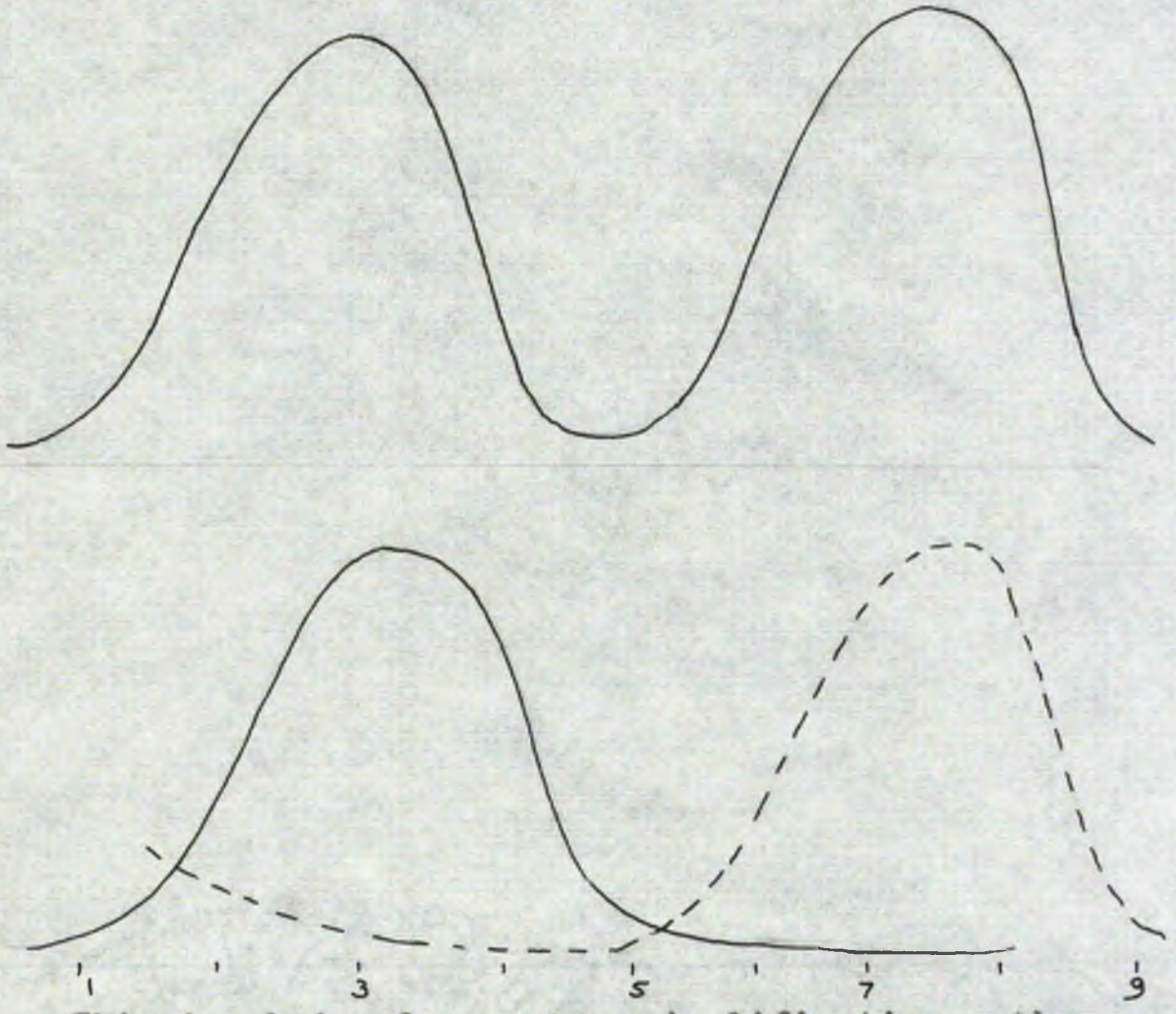
It is most interesting to find that in this post operative phase two maxima of amino acid excretion are usually seen and the urinary total nitrogens show concomitant changes. Presumably other urinary nitrogen metabolites (urea, uric acid, creatinine, imidazole derivatives, phenols, glycocyamine, allantoin indican and protein) may follow this pattern - especially those concerned in amino acid metabolism. If all thes factors show this "bactrian" trend then it is obviously a non specific effect of post-operative nitrogen metabolism rather than a specific effect of amino acid metabolism alone.

As to the reason for the necessity of these bactrian peaks it should be emphasized that they represent the sum total of excretory nitrogen metabolism. Therefore the two peaks may either be related to the same metabolic pathways or in fact represent two different phases in nitrogen metabolism.

In this latter case the second peak may

1) be dependent on the first nitrogen peak or

2) be independent, e.g.



This is obviously an over simplification, other smaller peaks remaining "hidden" in these greater changes.

Mention has been made of the circadian rhythms of plasma amino acids and adrenal cortical secretions. Whether this normal physiological pattern has any effect in the early post operative period or not is unknown at present. It is most likely to resume its normal pattern gradually during the first 10-12 days of post operative recovery.

It is well known that plasma fibrinogen and possibly other proteins are increased late in the post operative phase (10-12 days), and therefore may be partially responsible for changes occurring in this period in relation to wound healing and general nitrogen metabolism.

Allied information obtained during the investigation of these perfusions e.g. Haemoglobin and PCV values, electrolytes, creatinine etc. has been omitted from the Appendix as values were incomplete.

SUMMARY

1) The literature on biochemical changes in open cardiac surgery with especial reference to plasma protein and amino acids has been reviewed.

2) The changes that take place in plasma proteins during oxygenation of blood of open cardiac surgery have been investigated with emphasis on denaturation. This phenomena has been examined by the assay of blood and plasma sulfhydryl groups by different reagents and also viscosity and turbidity changes. The limitations of the two latter investigations has been discussed.

3) Haemolytic changes in plasma have been examined and the related importance of sulfhydryl groups discussed.

4) Plasma protein changes have been investigated by methods depending on net charge, shape, size, and molecular weight of individual molecules.

5) Although adrenal cortical response to open cardiac surgery has been investigated it has been pertinent to reinvestigate adrenal cortical secretion pari passu with amino acid data from blood and urine samples of patients not only because of their metabolic associations, but also because of the opportunity of comparing surgical patients receiving supplementary hydrocortisone throughout open

cardiac surgery with those not supplemented - the latter therefore depending on their own intrinsic secretion during surgical stress.

A review of relevant literature has accordingly been presented with emphasis on the difficulties involved in ACTH assay of plasma from surgical patients, and the practical advantages of 17-hydroxycorticosteroid assay in lieu of ACTH assays.

6) The plasma and urinary amino acid increase have been examined together in detail comparing not only the increase in each amino acid at each operation but also comparing the increase of an amino acid in all the operations investigated - thus obtaining mean values of each amino acid at regular intervals of time throughout each perfusion.

APPENDIX I

Haemoglobin Reagents

1) Saturated potassium persulphate. 7-8 gms of Analar Iron free $K_2S_2O_8$ in 100 mls. water. Kept in glass stoppered bottle 1-2°C. Shake well before use. Keep in refrigerator.

2) 10% Sodium tungstate. (Na_2WO_4): Iron free most important.

3) Standard iron solution - 0.702 gms ferric ammonium sulphate is dissolved in 100 mls. water and 5 mls. concentrated sulphuric acid added carefully. The mixture is gently warmed and concentrated potassium permanganate added drop by drop.

Transfer to 1 Litre flask and dilute to mark with water.

This solution contains 0.1mgm ferric iron/ml. and is stable.

4) 3 N Potassium thiocyanate (KCNS) 146 gm is dissolved in water and diluted to 500 mls. If turbid it is filtered. 20 mls. acetone may be added to improve keeping quality.

Haemoglobin Standard Iron Solution

(2)

2 mls. conc. sulphuric acid was carefully added to 25 mls. water in a 50 ml. graduated flask.

2 mls. saturated potassium persulphate and 2.5 mls. standard iron solution (0.1 mg Fe^{+++}/ml.) is added and mixed well, cooled and diluted to exactly 50 mls.

(3) Haemoglobin blank

25 mls. water and

2 mls. conc. sulphuric acid

2 mls. Sat. potassium persulphate diluted to

50 mls. exactly with water.

(4) Biuret Standard Gornall et al. 1949.

1st METHOD

1) 1.5 gm. Copper sulphate pentahydrate ($CuSO_4\ 5H_2O$)

2) 6.0 gm. Sodium potassium tartrate ($NaKC_4H_4O_6.4H_2O$)

3) These are dissolved in 100-200 mls. water and transferred to a 1 L. flask (volumetric) and 400-300 mls. water added.

4) A 10% solution of sodium hydroxide (300 mls.) is added to the above solution with constant stirring. The volume is made up to 1 L. and the reagent stored in a plastic or paraffin lined bottle.

The reagent keeps indefinitely but must be discarded on the appearance of a black or red precipitate.

2nd METHOD Biuret Reagent : Stock solution.

22.5 gms of sodium potassium tartrate is dissolved in 0.2 N sodium hydroxide and the volume made up to 200 mls. 7.5 gms of copper sulphate pentahydrate are added and when dissolved 2.5 gms of potassium iodide is added and the total volume made up to 500 mls. with the sodium hydroxide (0.2N).

Dilution of Stock.

20 mls. of stock solution are diluted to 100 mls. with 0.2 N sodium hydroxide and 0.4 gm. of potassium iodide added.

(5) Protein Standard

1 gm. of stock crystalline Bovine Albumin (Armour) was dried overnight over phosphorous pentoxide before weighing. This was dissolved in water next morning and made up to 100 mls.

The solution was preserved with a few drops of chloroform. A 0.1% solution is made from stock by diluting 10 mls. to 100 mls. with distilled water.

(6) Chromatographic Reagents

Anisidine

Following reaction with Ehlichs reagent, the chromatogram was dipped in the following anisidine solution, allowed to dry for 5 minutes and then dipped through 0.4 % NaOH. Colours appear immediately.

Anisidine Reagent

1) p. Anisidine, 1% in Ethanol, and 1% v/v concentrated HCl. -------- 1 Vol.

2) Amyl nitrite 2% in Ethanol -------- 1 Vol.

3) Sodium hydroxide N/2 (2%) in water

Solutions 1) and 2) are stable indefinitely. Prior to use they are mixed 1:1 ratio and allowed to stand 3 minutes.

Sulphanilic Acid

1) Sulphanilic acid, 9 gms., concentrated HCl, 90 mls. and water 900 mls. --------- 1 Vol.

2) Sodium nitrite 5% in water --------- 1 Vol.

3) Anhydrous sodium carbonate, 10% in water --------- 2 Vol.

The phenol retained on the phenolic chromatogram is eluted by ether or absolute ethanol prior to detection by this reagent, and the chromatogram dried as below.

Solutions 1 and 2 are mixed immediately before use and allowed to stand for 5 minutes at room temperature.

Solution 3 is then CAREFULLY added (much effervescence). The chromatogram should be oven dried and then dipped rapidly through the reagent and laid flat on a clean sheet of chromatography paper.

Nitroso-napthol Reagent.

1) α nitroso β napthol 0.1% in Ethanol 9 Vol.

2) Concentrated HNO_3 1 Vol.

Solutions 1 and 2 are mixed immediately before use, the chromatogram is passed through, hung up to dry for 2-3

minutes at room temperature, and then heated at 105°C for 2 minutes. It is important not to over heat the chromatogram, as the colours fade rapidly. The latter may be mitigated by passing the chromatogram through dilute ammonia.

Appendix 2.

Change in Plasma -SH during operations.

NEM method: mM/SH/L x 10^{-1}

	0	30	60	90	120	150	180	210	240 mins
1. J.M.	1·16	1·26	1·37	1·39	1·33	1·26	1·35	1·51	1·69
2. P.M.	1·27	2·03							
3. B.K.	1·08	1·13	1·26	1·398	1·53				
4. M.Ch.	0·95	1·151	1·32	1·5	1·62	1·7	1·82	1·827	
5. I.D.	1·43	1·442	1·48	1·563	1·65	1·75	1·88		
6. S.S.	1·0	1·01	1·03	1·052	1·04	1·252	1·56		
7. D.Mo.	1·6	1·573	1·56	1·54	1·617	1·74	2·03	2·36	
8. F.K.	1·075	1·275							
9. C.N	1·52	1·541	1·622	1·69	1·825	1·85			
10. R.M	1·076	1·094	1·127	1·193	1·335	1·484	1·61		
11. A.C.	1·6	1·748	2·043	2·222	2·31		2·33		
12. D.S.	1·016	1·35							
13. R.D	1·565	1·561	1·579	1·617	1·673	1·709	1·723		
14. H.D.	1·512	1·563							
15. J.McC	2·266	2·276	2·3	2·51	2·55	2·576	2·584		
16. K.R.	2·089	2·22	45 2·363						
17. D.My	2·13	2·232							
Melrose	0 1·78	1 1·852	2 2·02	3 2·05	4 2·251	5 2·648	6 3·32	7 3·653	8 Hours 4·49

Although in short operations blood samples were taken every 10 minutes, these results are not recorded above but can be seen on the appropriate graphs.

Change in Blood-SH during operations

DTNB method: mM/SH/L

	0	30	60	90	120	150	180	210	240
I.J.M.	2·871	2·89	2·943	3·18	2·975	2·91	2·952		
2. P.M	3·2	4·077							
3. B.K.	2·76	2·823	2·91		3·149	3·94			
4. M.Ch.	2·55	2·57	2·558		2·63		3·24	3·58	3·68
5. I. D.	2·948	3·15	3·53	3·88	4·157	4·371			
6. S.S.	3·0		3·051		3·819	4·476	4·71		
7. D.Mo.	3·59		3·6	3·583	3·57		3·82	4·319	3.35 4·73
8. F.K.	3·097	3·395							
9. C.N.	3·32	3·54	3·427	3·69	4·041				
10. R.M.	3·072	3·085	3·151	3·3	3·689	3·92	4·057		
11. A.C.	2·9	3·11	3·49	3·569	3·68	3·88			
12. D.S.	3·15	3·822	40 3·957						
13. R.D.	2·876	2·81	2·92	2·963	3·091	3·298	3·354		
14. H.D.	3·254	3·362	40 3·578						
15. J.McC	3·153	3·17	3·272	3·8	4·09	4·188	4·21		
16. K.R	3·35	3·578	40 3·75						
17. D.My	3·268	3·355							
Melrose	0 3·352	1 3·49	2 3·673	3 3·85	4 4·21	5 4·658	6 5·47	7 4·1	8 2·52	9 Hours 1·166

Table 3.1 Plasma Turbidity values during cardiac and non-cardiac operations.

	0	30	45	60	90	105	120	150	180	210	240 mins.
2 "P.M."	0·12	0·149									
8 "F.K."	0·101	0·131	0·145								
12 "D.S."	0·139	0·169	0·183								
14 "H.D."	0·136	0·161	0·17								
16 "K.R."	0·2	0·26	0·3								
17 "D.M."	0·11	0·13									
3 "B.K"	0·133	0·163	0·177	0·17	0·192		0·21				
5 "I.D."	0·104	0·118	0·13	0·143	0·162		0·165	0·167	0·168		
9 "C.N."	0·109	0·139	0·153	0·146	0·17		0·183				
1 "J.M."	0·25	0·29		0·34	0·41		0·39	0·31	0·27		0·44
4 "M.Ch."	0·102	0·13	0·146	0·16	0·19		0·213	0·236	0·26		
7 "D.Mo."	0·11	0·125	0·135	0·14	0·165		0·18	0·21	0·245	0·305	0·345
6 "S.S."	0·085	0·087	0·089	0·092	0·099		0·11	0·166	0·226	0·245	
11 "A.C."	0·135	0·16	0·17	0·2	0·25	0·275	0·275	0·268			
13 "R.D"	0·131	0·16	0·175	0·168	0·19		0·205	0·216	0·237		
10 "R.M."	0·105	0·16	0·18	0·205	0·215		0·22				
15 "J.McC."	0·133	0·159	0·168	0·199	0·248		0·273	0·275	0·28		
MEAN OF 14 CARDIAC CASES	0·12	0·15	0·164	0·157	0·18		0·194	0·205	0·226	0·278	0·345
MEAN OF 2 CONTROL OPERATIONS	0·122	0·145	0·168	0·199	0·25		0·273	0·275	0·28		
MELROSE	0·525	0·494	0·488	0·477	0·463	0·482	0·539	0·662	0·97	1·05	1·18	1·3
	0	30	60	90	// 2	3	4	5	6	7	8	9 hrs

Table 4.1 Viscosity changes @ 20°c.

gms protein/100mls plasma

			30	60	90	120	150	180	210	240	C	F
1·060	1. "J.M"	85·7 1·345	87·9 1·380	87·8 1·378	88·9 1·395	89·2 1·400	89·9 1·411	90·85 1·426			4·68	5·1
1·0122	2. "P.M"	83·8 1·31	84·5 1·326								4·42	4·48
1·024	3. "B.K"	87·8 1·378	88·2 1·384	89·1 1·398		89·9 1·411					4·77	4·8
1·0835	4. "M.Ch."	83·9 1·317	85·2 1·337	85·9 1·348	86·4 1·356	87·9 1·38	89·3 1·401	90·9 1·427			4·44	4·5
1·056	5. "I.D"	86·6 1·359	87·5 1·373	88·4 1·387	89·3 1·402	90·35 1·418	91·45 1·435				4·64	4·7
1·0976	6. "S.S"	92·0 1·444	92·7 1·455	94·0 1·475	95·35 1·497	97·2 1·526	100·05 1·570	104·0 1·63	101·0 1·585		5·26	5·7
1·0424	7. "D.Mo."	99·2 1·557	100·25 1·573	101·2 1·588	101·8 1·598	101·9 1·599	102·0 1·601	102·2 1·604	102·8 1·614	103·4 1·623	5·95	6·1
1·0126	8. "F.K."	90·9 1·427	92·05 1·445								5·23	5·5
1·0546	9. "C.N."	96·7 1·518	98·4 1·544	100·05 1·570	101·6 1·595	102 1·601					5·7	6·05
1·0692	10. "R.M."	87·5 1·373	88·4 1·387	89·45 1·404	90·9 1·427	92·75 1·456	93·3 1·464	93·5 1·468			4·9	5·4
1·0213	11. "A.C."	86·5 1·358	87·1 1·367	87·5 1·373	88·3 1·386	88·4 1·387	88·35 1·387				4·7	4·1
1·0198	12. "D.S."	87·0 1·365	88·7 1·392								4·7	5·1
1·0973	13. "R.D"	85·1 1·336	86·0 1·350	87·55 1·374	89·5 1·405	91·9 1·443	93·2 1·463	93·4 1·466			4·5	5·13
1·015	14. "H.D."	84·9 1·333	86·2 1·353								4·4	5·2
1·0414	15. "J.McC."	100·05 1·570	101·3 1·590	101·9 1·599	102·8 1·614	103·75 1·628	104·1 1·634	104·2 1·635			6·05	6·3
1·0039	16. "K.R."	97·3 1·527	97·65 1·533								7·0	5·7
·	17. "D.My."	·	·									
											5 hrs ↓	6 hrs ↓
	Melrose	98·9	99·6	99·7	100·1	99·95		99·9		100·2	100·6	100·9

Upper figure in each square = the viscosity of plasma in time 't' secs.

Lower figure in each square = the viscosity of plasma in centipoise units relative to water. i.e. relative viscosity.

Figures in left hand column = ratio of relative viscosity increase of final plasma / control plasma.

Plasma haemolysis during operation Table 5.6.

O.D. values 0·01 ≡ 198·6 mgms % Hb.

	0	30	60	90	120	150	180	210	240 mins.
1. J.M									
2. P.M.	0·0065	0·0095							
3. B.K.	0·0078	0·0082	0·0087	0·00945	0·011				
4. M.Ch.	0·005	0·0058	0·0135	0·02	0·025	0·029	0·0325	0·036	
5. I.D.	0·0047	0·005	0·009	0·012	0·016	0·0213			
6. S.S.	0·007	0·0088	0·0105	0·0125	0·014	0·02	0·025		
7. D.Mo.		0·0048	0·0068	0·0088	0·0124	0·016	0·022	0·03	0·036
8. F.K.	0·007	0·01							
9. C.N.	0·011	0·019	0·0263	0·032	0·0363	0·034			
10. R.M.	0·0063	0·0094	0·0136	0·0159	0·0213	0·0237	0·028		
11. A.C.	0·0022	0·0028	0·0055	0·0109	0·0134	0·014	0·0141		
12. D.S.	0·004	0·0063							
13. R.D.	0·018	0·019	0·024	0·0241	0·0245	0·028			
14. H.D.	0·0067	0·0075							
15. J.McC	0·0065	(0·245)*	0·0113	0·009	0·012	0·0113	0·125		Control Operation
16. K.R.	0·0063	4.5 0·0075							
17. D.My.	0·0067	0·007							Control operation
Mean	0·0067	0·0088	0·0115	0·0155	0·0187	0·022	0·0223	0·033	0·036
Max	0·018	0·019	0·0263	0·032	0·0363	0·034	0·0325	0·036	
min	0·0022	0·0028	0·0055	0·0088	0·011	0·0113	0·0125	0·03	

The above factors × 19,860 ≡ mgms Hb/100mls.

* Owing to the fracture of a centrifuge tube during centrifugation this specimen was grossly haemolysed

Plasma Amino acids. mgms/100mls.

"P.M. 2."

	Control	Final 30mins.
Aspartic Acid	2·3	10·7
Threonine	2·9	7·8
Serine	1·72	7·83
Glutamic Acid	3·9	18·6
Proline	2·7	6·38
Glycine	1·92	5·27
Alanine	3·18	9·8
Valine	3·2	7·3
Cysteine	–	–
Methionine	–	–
Isoleucine	–	0·3
Leucine	4·1	12·9
Tyrosine	1·05	3·33
Phenylalanine	1·6	7·8
Hydroxylysine	–	0·78
Ornithine	–	0·94
Lysine	3·8	20·73
Histidine	2·1	5·68
Arginine	2·33	6·37
NH_3	–	14·3

"3.B.K."

Control	1	Final 2hrs.
3·5	10·9	12·5
2·25	8·48	8·6
2·43	6·43	7·6
15·25	30·91	38·4
2·44	5·3	7·21
2·07	5·89	6·5
3·3	10·51	12·00
3·35	9·07	10·8
0·3	0·137	0·12
–	–	0·81
0·68	1·87	2·24
7·5	21·2	20·1
1·5	4·06	4·32
2·03	8·41	9·52
0·6	1·47	1·61
0·79	0·95	0·92
6·5	13·33	14·1
1·5	11·07	12·14
1·5	5·07	5·8

Plasma Amino acids mgms/100mls

"4 M.Ch."

	Control	1½hrs	Final 3hrs.
Aspartic Acid	2·15	33·7	45·7
Threonine	2·28	21·33	28·0
Serine	1·68	16·7	22·8
Glutamic Acid	15·3	61·2	84·3
Proline	3·8	17·5	21·0
Glycine	2·66	12·46	16·5
Alanine	3·8	29·9	39·35
Valine	3·9	32·6	37·0
Cysteine	–	8·57	13·5
Methionine	–	3·67	4·2
Isoleucine	1·2	5·82	7·0
Leucine	5·62	40·27	61·3
Tyrosine	0·4	15·78	18·17
Phenylalanine	2·6	32·6	37·34
Hydroxylysine	0·3	0·38	0·77
NH_3	4·8	–	25·7
Ornithine	0·73	0·93	1·41
Lysine	6·5	42·6	52·2
Histidine	2·0	20·5	28·8
Arginine	1·44	4·81	9·6

"5 I.D"

Control	1 hr.	Final 2hrs.
0·5	9·8	13·06
1·7	7·33	8·3
1·12	6·9	7·7
2·0	12·6	28·8
2·45	5·37	6·95
1·65	8·03	7·95
3·6	11·4	12·6
2·85	11·26	13·06
1·2	0·92	0·61
0·6	0·83	0·52
1·3	2·12	1·63
1·8	15·8	23·55
1·25	4·37	4·97
1·1	8·36	9·13
–	0·96	1·5
–	8·7	—
0·72	0·75	0·7
2·8	10·96	12·4
1·3	7·9	8·3
1·9	5·77	6·2

Plasma Amino acids mgms/100mls

	"6.S.S"			"7. D.Mo."			
	Control	1 hr	Final 3hrs.	Control.	1 hr	3 hrs	Final 3½hrs.
Aspartic Acid	1·93	10·6	14·03	1·93	12·6	23·41	32·5
Threanine	1·47	7·03	8·53	1·47	10·3	20·07	31·73
Serine	1·67	5·7	8·0	1·67	9·63	17·16	23·4
Glutamic Acid	8·6	18·8	34·23	6·7	20·1	36·22	51·7
Proline	2·3	6·93	7·55	2·31	4·73	18·47	26·73
Glycine	2·11	6·4	7·8	2·11	5·7	15·3	23·6
Alanine	2·8	10·04	14·77	2·79	11·87	29·7	44·6
Valine	2·88	10·73	14·1	2·9	11·8	25·42	34·37
Cysteine	1·19	3·67	10·91	2·2	5·82	—	13·8
Methionine	—	0·3	0·76	—	0·82	3·98	4·3
Isoleucine	1·31	1·51	1·43	1·3	4·6	8·8	13·68
Leucine	4·45	16·96	22·27	4·45	21·3	40·12	77·6
Tyrosine	0·91	4·31	4·45	0·91	6·18	11·26	16·33
Phenylalanine	1·69	7·1	10·4	1·7	14·2	23·75	35·6
Hydroxylysine	—	0·8	1·2	0·35	0·88	1·23	1·3
NH_3	—	3·9	—	—	—	—	23·37
Ornithine	0·63	1·4	1·11	0·6	—	1·03	1·43
Lysine	3·76	9·83	17·6	3·8	17·7	47·8	73·6
Histidine	1·64	5·74	8·5	1·65	8·3	12·7	18·63
Arginine	-	5·73	10·2	3·0	8·64	14·93	27·8

Plasma Amino acids mgms/100mls

"8. F.K."

	Control	Final 45mins
Aspartic Acid	2·1	27·5
Threonine	2·56	17·2
Serine	1·92	13·0
Glutamic Acid	13·8	53·5
Proline	3·6	13·6
Glycine	2·52	15·0
Alanine	3·8	22·0
Valine	3·16	1·2
Cysteine	–	–
Methionine	–	–
Isoleucine	0·8	0·3
Leucine	5·13	42·0
Tyrosine	0·87	9·0
Phenylalanine	3·4	21·4
Hydroxylysine	0·44	0·75
NH_3	–	–
Ornithine	1·6	1·79
Lysine	3·47	24·3
Histidine	3·7	18·4
Arginine	2·44	6·7

"9.C.N."

Control	1 hr.	Final 2 hrs.
2·2	11·6	14·9
2·97	7·67	8·92
1·48	5·1	7·48
9·6	16·68	33·13
3·8	5·82	7·41
2·66	5·77	6·9
3·23	10·46	12·71
3·42	9·97	13·1
0·67	–	6·7
Tr.	–	0·48
1·3	2·16	2·0
5·3	17·41	25·6
0·4	3·8	4·92
2·63	8·4	9·98
–	0·83	–
–	5·6	–
0·98	1·15	0·73
6·2	11·7	15·8
2·0	5·7	6·3
1·48	5·73	5·9

Plasma Amino acids. mgms/100mls

"10. R.M."

	Control	30	60	90	Final. 160 mins.
Aspartic Acid	2·15	8·97	11·1	15·7	36·1
Threonine	2·3	7·1	9·2	12·63	19·7
Serine	1·7	7·03	8·47	8·5	17·7
Glutamic Acid	15·3	27·5	29·0	35·7	71·2
Proline	3·8	4·8	5·1	6·13	22·5
Glycine	2·7	3·95	4·8	5·7	15·4
Alanine	3·8	8·4	10·0	10·97	41·1
Valine	2·83	7·6	12·0	14·7	38·5
Cysteine	0·6	—	4·9	—	11·2
Methionine	—	—	1·5	—	3·7
Isoleucine	1·2	1·7	2·5	5·33	11·6
Leucine	5·62	20·2	29·9	39·8	86·4
Tyrosine	0·4	4·53	5·6	6·96	12·15
Phenylalanine	2·6	9·76	11·63	15·4	30·4
Hydroxylysine	0·38	0·51	0·48	0·6	0·78
NH_3	—	14·2	—	—	19·3
Ornithine	—	0·19	0·24	0·5	0·71
Lysine	6·5	25·9	39·1	52·7	99·0
Histidine	2·0	7·37	9·87	9·93	14·4
Arginine.	1·44	10·6	14·5	18·9	35·3

Plasma Amino acids mgms/100mls

"H.A.C."

	Control	½ hr.	1	2	Final. 3 hrs
Aspartic Acid	2·97	8·98	12·3	9·4	10·92
Threonine	2·6	6·26	4·75	5·88	4·31
Serine	2·19	5·51	3·35	5·37	4·1
Glutamic Acid	12·3	24·2	32·9	21·9	24·0
Proline	2·74	6·54	8·73	7·32	11·22
Glycine	2·55	4·94	6·2	5·6	6·9
Alanine	4·24	8·8	12·25	10·97	12·85
Valine	5·25	11·48	11·03	8·9	12·2
Cysteine	—	2·36	—	—	9·73
Methionine	—	—	—	1·82	1·5
Isoleucine	0·83	1·9	1·25	1·25	0·44
Leucine	8·7	22·8	18·1	22·6	18·0
Tyrosine	1·55	3·9	3·5	3·15	3·3
Phenylalanine	3·83	8·15	7·7	8·5	9·04
Hydroxylysine	0·61	0·575	0·67	0·52	1·0
NH_3	—	2·86	7·44	6·75	7·14
Ornithine	—	1·17	—	0·523	0·97
Lysine	3·43	6·44	18·0	28·8	16·0
Histidine	1·9	—	1·08	15·1	4·3
Arginine	2·2	—	3·9	9·77	4·4

Plasma Amino acids mgms/100mls

	"12. D.S."		"13. R.D."				
	Control	Final 45 mins.	Control				
Aspartic Acid	2·4	8·73	2·92	7·7	14·9	10·2	21·75
Threonine	3·13	7·87	1·77	5·25	8·5	7·2	11·2
Serine	1·92	8·26	1·89	4·4	7·17	6·3	8·95
Glutamic Acid	3·13	28·4	10·5	21·9	36·1	31·1	36·55
Proline	1·94	5·24	1·68	5·63	7·46	7·64	7·96
Glycine	2·4	4·92	2·67	5·47	9·53	9·22	11·98
Alanine	3·37	9·4	3·8	15·57	15·1	11·4	20·87
Valine	4·03	13·3	3·3	10·12	12·3	10·37	22·7
Cysteine	—	—	1·82	—	5·6	8·32	10·5
Methionine	0·54	0·6	—	0·58	—	1·9	2·6
Isoleucine	0·9	1·6	0·71	0·49	—	1·35	2·9
Leucine	3·2	31·5	7·4	18·2	29·6	27·63	35·2
Tyrosine	1·49	7·35	1·66	3·83	4·52	4·0	4·83
Phenylalanine	1·91	13·2	3·9	8·33	8·6	8·8	12·4
Hydroxylysine	0·57	0·75	—	—	—	1·57	2·43
NH_3	—	18·04	5·92	10·67	13·12	—	12·48
Ornithine	0·43	1·09	0·34	0·61	1·17	1·33	0·92
Lysine	3·2	31·7	3·33	9·3	13·3	22·9	51·4
Histidine	1·02	10·21	Tr.	—	9·4	15·8.	—
Arginine	1·56	13·72.	3·0	7·87	8·48	10·6	25·27

Plasma Amino acids. mgms/100mls

	"14. H.D."		"15. J.McC."				
	Control	Final 45mins	Control	½ hr.	1	2	Final 3hrs
Aspartic Acid	2·3	17·0	0.66	5·77	6·07	8·5	7·87
Threonine	2·13	14·93	1·64	6·31	6·48	6·0	7·68
Serine	2·51	13·3	1·07	6·54	7·22	6·44	7·12
Glutamic Acid	6·37	39·7	2·4	16·5	20·7	26·9	14·55
Proline	3·62	12·66	2·25	5·71	5·52	8·57	6·83
Glycine	2·12	17·63	1·58	3·72	3·76	5·2	5·07
Alanine	2·8	18·37	3·6	4·09	4·71	8·44	8·74
Valine	2·96	14·32	2·84	4·56	4·88	8·96	9·07
Cysteine	—	—	0·61	—	3·53	—	6·81
Methionine	0·64	1·9	—	0·54	0·575	—	0·61
Isoleucine	1·3	2·03	1·23	1·19	0·91	1·5	1·72
Leucine	4·41	29·97	1·83	7·32	8·96	17·8	18·14
Tyrosine	3·01	7·34	1·17	1·71	1·91	2·4	3·3
Phenylalanine	3·79	17·08	1·2	3·4	3·67	6·72	6·42
Hydroxylysine	0·41	1·67	0·25	1·11	1·51	0·98	1·23
NH_3	—	—	—	—	6·3	9·11	6·46
Ornithine	—	0·69	0·8	0·97	1·2	—	1·32
Lysine	2·81	20·08	3·32	16·6	17·0	23·56	29·3
Histidine	1·92	16·77	1·3	4·99	6·45	2·17	5·7
Arginine	1·43	6·4	1·93	—	8·37	7·43	8·2.

Plasma Amino acids. mgms/100mls

	"K.R. 16."		"17. D.My."		Melrose Experiment	
	Control	Final 45mins			Control	Final.
Aspartic Acid	2·13	10·2			4·11	5·49
Threonine	1·96	9·06			2·82	4·3
Serine	1·57	8·67			2·74	3·68
Glutamic Acid	5·03	25·8			13·67	15·37
Proline	3·2	5·33			2·73	5·56
Glycine	2·71	4·46			1·44	3·35
Alanine	3·8	8·94			4·13	6·88
Valine	3·77	10·77			3·9	7·05
Cysteine	—	—			0·57	3·0
Methionine	—	0·97			—	Tr.
Isoleucine	1·09	2·32			0·74	1·19
Leucine	3·02	23·84			4·01	9·97
Tyrosine	1·66	5·36			1·84	2·62
Phenylalanine	1·74	12·57			3·09	4·7
Hydroxylysine	0·31	0·75			—	—
NH_3	—	—			3·06	6·37
Ornithine	—	0·44			1·1	—
Lysine	4·67	23·54			7·92	10·07
Histidine	1·73	9·22			2·12	4·25
Arginine	2·63	9·43			3·0	2·6·

"4. M. Ch." Urinary Amino acids. mgms/24 hrs.

	Pre. op.	Post op	2	3	4	5	6	7
Aspartic Acid	75	53·5	282	221	273	160	261	242
Threonine	29	34	159	132	155	81	150	183
Serine	35	24	143	196	154	71	144	146
Glutamic Acid	281	143	312	400	548	396	786	719
Proline	33	29·5	144	150	159	74	235	215
Glycine	422	97·5	315	865	241	197	257	555
Alanine	61	37·5	187	294	179	117	123	213
Valine	45·5	36·5	619	446	200	320	395	407
Cysteine	20	124	337	299	238	218	225	229
Methionine	62	–	67	61	48	–	19	–
Isoleucine	10	11·25	83	130	86	64	82	25
Leucine	25	36	235	285	259	164	136	225
Tyrosine	23	14	117	263	142	77	88	66
Phenylalanine	34	22	170	428	184	112	90	86
Hydroxylysine	–	–	–	–	–	–	–	–
Ornithine	–	–	–	–	–	–	–	–
Lysine	44	31	199	292	328	155	124	141
Histidine	67·5	16·5	115	494	161	87	130	118
Arginine	29	18	132	171	83	105	58	173
Total mgms/24hrs	1235	728	3555	5065	3388	2397	3284	3743

"7 D.Mo." Urinary Amino acids. mgms/24hrs

	Pre op 2.	Pre op. 1.	0	1	2	3	4	5
Aspartic Acid	123	142	89	142	553	167	314	170
Threonine	41	29	20	69	270	65	144	50
Serine	49	54	37	76	280	71	144	53
Glutamic Acid	247	400	265	658	1619	662	932	137
Proline	46·5	71	33	70	280	79	129	55
Glycine	283	360	350	197	592	267	436	270
Alanine	39	39	35	98	384	92	97	119
Valine	25	"148"	55	110	313	74	216	153
Cysteine	17	10·5	11	216	397	363	303	114
Methionine	16	12	51	33	53	46	22	–
Isoleucine	19·6	15·5	12	41	117	19	51	33
Leucine	20·5	23	14	40	418	85	197	86
Tyrosine	28	33	40	207	142	45	82	14
Phenylalanine	32	25	14	567	303	78	127	19
Hydroxylysine	n/a	–	–	–	–	–	–	–
Ornithine	n/a	–	–	–	–	–	–	–
Lysine	28	53	42	144	387	83	99	109
Histidine	138	111	92	116	197	106	98	108
Arginine	69	12	15	36	221	39	114	55
Total								

"15. J. McC." Urinary Amino acids. mgms/24hrs.

	Pre Op.	Post Op. 1	2	3	4	5	
Aspartic Acid	120	110	314	335	158	150	
Threonine	47	31	169	131	94	89	
Serine	61	72	194	151	129	117	
Glutamic Acid	351	247	1502	1180	669	383	
Proline	56	68	409	194	160	75	
Glycine	212	239	405	151	211	261	
Alanine	44	38	225	101	128	128	
Valine	33	51	153	207	173	106	
Cysteine							
Methionine	39	62	181	313	270	273	
Isoleucine	17	27	45	57	44	31	
Leucine	23	38	73	104	22	100	
Tyrosine	37	55	95	58	80	81	
Phenylalanine	18	33	103	62	98	100	
Hydroxylysine	11	13	52	23·5	34	38	
Ornithine	1·6	0·9	2·1	3·2	4·4	4·0	
Lysine	83	92	163	318	401	417	
Histidine	73	90	149	225	240	343	
Arginine	32	49	85	42	92	75	
Total mgms/24hrs							

APPENDIX 3

Data on Patients

All the following patients were perfused with blood which was oxygenated by the Ryggs bag method. Usually only one of which was used. In perfusions of expected long durations two bags were sometimes used in parallel.

There was little variation in the drugs used but it is important to mention that hydrocortisone was routinely given in perfusions up and including '10 R.M.' but was witheld thereafter during the perfusions.

The cardiac abnormalities were either congenital septal defects or acquired valvular lesions. The latter were usually or rheumatic origin although one '7 D.M.' was syphilitic in origin.

Calculated blood flow rate. The rate in average adults was about 3.0 - 3.6 litres/min. In children it was of the order of 1.6 - 3.0 litres/min. The figure depends on blood volume which is best estimated from the surface area rather than weight.

In adults the B.F.R. = 2.4 x Surface area

"children the B.F.R. = 2.6 x Surface area.

Oxygenation rate. In children approximately 2.6 l/min/sq. metre and in adults 2.4 L/min/sq. metre. The gas used was 95% O_2 : 5% CO_2.

Urine In many perfusions there was often oliguria and not unfrequently 'apparent' anuria due to low renal blood flow, kinked or blocked catheters. Urine collections were thus few and far between. Analyses on these showed low amino acid and total nitrogen content.

Temperature The perfusate temperature was usually reduced slightly during perfusions. Two, '8 F.K.' and '12 F.K.' were reduced to 31 and 33°C respectively.

'1 J.M.' ♀. Age 38 Yrs. M.V.R.

Weight 6 st. 12 lbs. Calc. blood flow rate = 3.2 L/min.

Height 5' 1

Surface Area. 1.3 sq. m.

Total full perfusion time (F.P.T.) = 3½ hours.

Drugs: 'Mannitol' 250 mgms in 25% soln. hydrocortisone 100 mgms/hrly. heparin (8,000 units/45 mins.)

This particular perfusion had the disadvantage - from the point of view of research on blood changes - of having considerable blood added to the perfusate volume over the 3½ hours. 11.97 litres of blood and 6.84 litres of dextrose saline were added against a loss of at least 3.2 litres. These changes are reflected in the results shown in various sections.

No urine studies.

'2 P.M.' ♂. Age 10 Yrs. A.S.D.

Weight 65 lbs. Calc. flow rate = 2.84 L/min.

Height 4' 6"

Surface area 1.06 sq.m.

Total F.P.T. 30 minutes.

Drugs as above.

No urine studies.

'3 B.K.' ♂ Age 4 Yrs. A.S.D. and V.S.D.

Weight 33 lbs. Calc. flow rate = 1.64 L/min.

Height 3' 2"

Surface Area 0.63 sq. m.

Total F.P.T. 2 hours.

Drugs - as in '1 J.M.'

No urine studies.

'4 M.Ch' ♀ Age 52 years. A.V.R.

Weight 6 st. 13 lb.

Height 4' 7"

Surface Area 1.25 sq.m.

Total F.P.T. 3½ hours.

Drugs - as above plus isoprenaline and pot. chloride.

This long perfusion study was aided by the availability of post operative urines in which the amino acid excretion and total nitrogens were able to be assayed. It is of great interest and considerable importance that this patient was unconscious for the first 3-4 and 5th - 8th days post operatively for no overt clinical reason. She was conscious for 1 day only in the first week, the 4-5th day. The amino acid and total nitrogen excretion followed a similar course with maxima (bactrian) on the 3rd and 6th days. This

is discussed in the amino acid section. This patient recovered after a pulmonary complication one week post operatively.

'5 I.D.' ♀ Age 35 AVR + MVR

Weight 8 st. 12 (56.5 Kg.) Calc. flow rate 3.75 L/Min.

Height 5' 2"

S. Area 1.56 sq. m.

Total F.P.P. 2 hours.

Drugs: As in '1 J.M.' plus Arfonad (hypotensive agent)

No urine studies.

'6 S.S.' ♀ Age. 56 A.V.R.

Weight 53 Kg. C.F.R. - 3·53 L/min.

Height 4' 11"

S. Area 1·47 sq. m.

F.P.P. 3 Hours.

Drugs : As in '1 J.M.'

No urine studies.

'7 D. Mor' ♂ Age 51 A.I. : 2 Ryggs bags used.

Weight 70 Kg. C.F.R. Full flow (100 Revs.)

Height 5' 10"

S. Area -

F.P.P. - 3½ hours.

Drugs: As in '1 J.M.' plus Arfonad.

Post-operative Urinary amino acids and total nitrogens were investigated.

'8 F.K.' ♂ Age. 42. M.S. and I.

Weight 55 Kg. C.F.R. - 3·7 L/min

Height 5' 2½"

S. Area 1·55 sq.m.

E.P.P. 30 minutes (partial - 60 minutes).

Drugs : As in '1 J.M.' plus E.A.C.A. (epsilon amino caproic acid) antifibrinolytic agent 4 gm. init. + 1 gm/hrly. arfonad 250 mgm/100 mls and aramine (both hypertensive agents), isuprel (isoprenaline) 5 µg/ml, calcium chloride.

No urine studies.

The blood perfusate temperature was lowered to 31°C (87.8°F) during full perfusion.

'9 C.H.' ♀ Age. 46 M.I.

Weight 6 st 10 lb C.F.R. - 3·26 L/min.

Height 5' 1"

S. Area. 1·36 sq.m.

E.P.P. 2 hours.

Drugs. As in 1 J.M.

Very little extra blood was added throughout this perfusion.

'10 R.M.' ♂ Age 48 M.V.R.

Weight 7 st. 8 lb. C.F.R. 3.65 l/min.

Height 5' 5½"

S.A. 1.52 sq. m.

F.P.T. 3 hours.

Drugs. As in '1 J.M.' plus E.A.C.A. 30 mgm stat. and 20 mgms. hourly.

'11 A.C.' ♀ Age 43 M.V.R. 'Double Ryggs'.

Weight 8 st. 6 lb. C.F.R. -

Height 5' 1"

S. Area 1.42 (1.52) sq. m.

F.P.T. 2¾ hours.

Drugs. Heparin only: Hydrocortisone was not given in this perfusion for reasons described in the hormonal and amino acid sections.

There was good urinary flow in this perfusion. 50 mls. in the first ½ hour and 90 mls. in the following 2¼ hours.

'12 J.S.' ♂ Age 38 A.S.D.

Weight 17 st. C.F.R. - 5.9/L/Min.

Height 6' 7" ! (100 mls./Sec.)

S. Area 2.45 sq. m.

F.P.T. 45 mins.

Drugs: Heparin only No hydrocortisone.

The temperature of perfusate volume was reduced to 33°C (91.4°F) during the major procedure.

'13 R.D.' ♂ age 32. A.V.R. 'Double Ryggs'

Weight 11st. (70 Kg.) C.F.R. = 4.32 L/Min.
Height 3'6"
S. Area 1.8 sq. m.
F.P.T. 2¾ hours.

Drugs. Heparin only.

There was a good urinary flow during perfusion, 245 mls. in the first 2 hours and 130 mls. in the last hour. Small post operative urine aliquots enabled total nitrogens to be assayed but were insufficient for amino acid studies.

'14 H.D.' ♀ Age 15 A.S.D.

Weight 8 st 7½
Height 5' 2" C.F.R. = 3.3 L/Min.
S. Area 1.3 sq. m.
F.P.T. 45 minutes.

Drugs. Heparin.

'15 J.Mc' ♂ Age 46 Oesop. Ca + Hist. Hernia.

Weight n/d.

Height n/d.

This case was used as a control and is remarkable in that it lasted an unusual duration of 3 hours and is thus an interesting comparison to the long perfusion results.

Post operative urines were obtained and amino acid and total nitrogen content assayed.

'16 K.R.' ♂ Age 5 Yrs. A.S.D.

Weight 38 lbs. C.F.R. 1.92 L/Min.

Height 3' 8"

S. Area 0.74 Sq. m.

F.P.T. 45 minutes.

Small aliquots of post operative urines were obtained which enabled the total nitrogens to be done, but these urines were not consecutive enough to allow a comprehensive amino acid analysis to be obtained.

'17 D.M.' ♂ Age 65 years. Ant. resection of colon (Ca)

Duration of operation 45 minutes :

This was a short control abdominal operation. Blood specimens only were obtained.

FINAL CONCLUSION

1) Minimal denaturation of plasma proteins appears to take place during cardiac perfusions using sulfhydryl analysis, turbidity, viscosity, ultracentrifugal, and electrophoretic methods.

2) The use of hydrocortisone (100mgms. hrly.) during perfusions increases plasma amino acid concentrations up to 100 mgms/100 mls.

When hydrocortisone is witheld - the plasma amino acids increase to 30 - 50 mgms/100 mls.

Since all amino acids are toxic to a varying degree limitation of extrinsic hydrocortisone during perfusion may well be beneficial.

The increase in plasma amino acids is roughly proportional to the duration of the perfusion up to 3 hours after which the increase is more severe.

There is no relationship between the degree of haemolysis and the plasma amino acid concentration.

Lysine (one of the four most toxic amino acids) was greatly increased in the plasma during perfusion, and is also greatly increased during ACTH therapy. The fact that histidine and arginine are increased during perfusion in the moderate range (but are not increased in ACTH therapy) suggests that there is another cause for the increase in plasma amino acids during perfusion, such as Hepatic

dysfunction. These findings would also support the increase in tyrosine, phenylalanine and threonine concentrations found during perfusion in the "moderate increase range". Ankeney, 1961, uses the increase in these three amino acids as parameters of hepatic dysfunction. Other possible causes have been discussed.

Of the four most toxic amino acids - lysine has been mentioned, the other three are tyrosine, histidine and tryptophan.

Tyrosine and histidine were increased in moderate concentrations, and tryptophan hardly at all.

Arginine was increased in the moderate concentration range and would therefore be expected to have some extra protective effect against the toxicity of other amino acids (see Guillino et al. 1950 and 1956).

3) The maximum urinary excretion of amino acids appears in two waves. The first between the 2nd and 4th and the second wave between the 4th and 6th post operative days. The urinary total nitrogen results complement these findings. The difference in the increased urinary amino acid excretion is greatly influenced by (a) The relative plasma amino acid concentration. (b) Factors affecting renal function.

B I B L I O G R A P H Y.

A. Academic Press. Sulfur in Proteins p. 75, 1959.

Albanese A. J. Biol. Chem. 157 613.

Alexander, N.M. Anal. Chem. 30 1292, 1958.

Ankeney, J.L., Dimlich, H, Nash, C.L. & Carter T. Surg. Forum. 12 209 1961.

Anson, M.L., Mirsky, A.E. J. Gen. Physiol. 9 169, 1925.

_____ J. Phys. Chem. 35 185, 1931.

Archibald, R.M. J.B.C. 154 643, 1944.

Astrup, T., Stage, A., Olsen, E. Acta Chem. Scand. 5 1343, 1951.

B. Baer, D.M., Osborne, J.J., Perkins, H.A., Melrose, D.G., Norman, A., Gerbode, F. Amer. Heart J. 56 652, 1958.

Benesch, R., Benesch, R.E. Arch. Biochem. 19 35, 1948.

_____ Arch. Biochem. 28 43, 1950.

Benesch, R. Science 123 17 1938.

Benesch, R., Benesch, R.E. J. Biol. Chem. 236 405, 196

_____ J. Amer. Chem. Soc. 73 3391 1951.

Benesch, R.E., Benesch, R. J. Phys. Chem. 56 648, 1952

Berson, S.A., Yalow, R.S. J. Clin. Invest. 47 2725, 1968.

Bhattacharya, S.K., Robson, J.S., Stewart, C.P. B. Chem. J. 60 696, 1954.

Biench, R., Kalle, K. Z. Physiol. Chem. 175 115, 1928.

Binet, L., Weller, G. Bulletin Soc. Chim. Biol. 16 1284, 1934.

Björk, V.O., Catoliotti, F., Intonti, F. J. Cardiovas. Surg. 2 488, 1961.

Bowman, R.L., Udenfriend, S. Science In Press. 1957.

Boyer, P.D., J. Am. Chem. Soc. 76 4331, 1954.

Boyer, P.D., Lardy, H.A., Myrback, K. The Enzymes Vol. I Acad. Press. 1959.

Bull, H.B., J. Biol. Chem. 123 17, 1938.

Bumpus, F.M., Page I.H. J. Biol. Chem. 212 111, 1955.

Burstone, M.S., Falk, J.E. J. Histochem, 4 217, 1956.

C. Campbell, H.A., Mushburn, L.T., Boyse, E.A. Old L. J. Biochem. 6, no. 3. March '67.

Casey, J.H., Zimmerman, B. Surgery, Gynae + Obs. 105 179, 1957.

Cavallini, D., Graziani, M., Dupre, S. Nature 212 294 1966.

Cecil, R., McPhee, J.R. Adv. Prot. Chem. 14 255, 1959.

Chytil, F., Ceskovslov. Gastroenterol. Vyziva 10 244 1956.

Clark, C.T., et al., J. Biol. Chem. 210 139, 1954.

Clark, I. J. Biol. Chem. 200 69, 1953.

Cole, R.D., Stein, W.H., Moore S. J. Biol. Chem. 233 1359, 1958.

Colowick, S.P., Kaplan, N.O. Methods in Enzymology IV. 256, 1957.

Consden, R., Gordon, A.H., Martin, A.J.P. Biochem. J. 41 590, 1947.

Cooper, C.E., Nelson, D.H. J. Clin. Invest. 41 1599, 1962.

Cramer, A., Frank, R., Winnick, M., Theodore, J. J. Biol. Chem. 150 259, 1943.

Crosby Munn Furth., V.S. Armed Forces Med. J. 5 693, 1954.

Cuthbertson, D.P., Lancet, I., 433, 1942. Mammalian Protein Metabolism: Ed. Munro H.N., Allison, J.B. Vol. II 1964. Acad. Press.

Cuthbertson, D.P., Thomsett, S.L. Brit. J. Exp. Path. 16 471, 1935.

D. Davis, B. Personal Communication. Imp. Canc. Res. Fund. Hill Hill. London N.W.7.

Davis, J.W.L., Ricketts, C.R. Bull. J.P. Lancet I. 346 1959.

Dent, C.E., Schilling, J.A. Biochem. J. 44 318, 1949.

Dennis, C., Spreng, D.S., Nelson, G.E., Karlson, K.E., Nelson, R.M., Thomas, J.V. Eder. W.P., Varco, R.L. Ann. Surg. 134 709, 1951

De Wall, R.A., Warden, H.E., Gott, V.L., Read, R.C., Varco, R.L., Lillehei, C.W. J. Thor. Surg. 32 591, 1956.

_____ J. Amer. Med. Ass. 165 1788, 1957.

Dillard, G.H.L., Brecher, G., Cronkite, E.P. Proc. Soc. Expt. Biol. Med. 78 853, 1951.

Dimililer, I., Trout, R.G., J. Thor. & Cardiovas. Surg. 48 1964.

_____ Circ. Supp. 4 1965.

Duggan, E.L., Giese, A.C., Luck, J.M. Arch. Biochem. Biophys. 28, 1950.

Duggan, E.L., Udenfriend, S. J. Biol. Chem. 223 313, 1956.

E. Eades, C.H., Pollack, R.L. J. Nat. Cancer. Inst. 15 421, 1954.

Eades, C.H., Pollack, R.L., Hardy, J.D. J. Clin. Invest. 34 1756, 1953.

Edelhoch, H.E. et al., J. Am. Chem. Soc. 75 5058, 1953.

Editorial. Nutrit: Revue 16 73, 1958.

Einerth, Y., Hedner, P., Wicklander, O. Acta Chir. Scand. 130 411, 1965.

Ellman, G.L. Arch. Biochem. & Biophys. 82 70, 1959.

Ellman, G.L. Arch. Biochem. & Biophys. 74 443, 1958

Engel, F.L., Winton, M.G., Long, C.N.H. J. Exp. Med. 77 397, 1943.

Engel, F.E., Harrison, H.C., Long, C.N.M. J. Exp. Med. 79 9, 1944.

Evered, D.F. Ph.D. Thesis London University, 1954.

Everett, M.R. Medical Biochemistry, New York, 1946.

F. Feigin, R.D., Dangerfield, H.G., Beisel, W.R. Nature 221 94, 1969.

Feitelson, J., Partridge, S.M. Biochem. J. 64 607, 1956

Fishman, W., Govier, W.M. Science 101, 277, 1945.

Fleck, A., Munro, H.N. Metab. Clin. Exp. 12 783, 1963.

Flodin, P., Killander, J. B.B.A. 63 403, 1962.

Folin, O., Wu, H. J. Biol. Chem. 38 81, 1919.

Forsham et al., J. Clin. Endocrin. 8 15, 1948.

Fraenkel, Conrat, H., et al., J. Am. Chem. Soc. 73, 625, 1951.

Friodovich, I., Handler, P. Anal. Chem. 29 1219, 1957.

Friedberg, F., Greenberg, D.M. 168, 405, 1947.

Friedman, E., Marrian, D.H., Simon, Reuss, I. Brit. J. Pharmacol. 3, 335, 1948.

_____ Brit. J. Pharmacol. 4 105, 1949.

Friedman, E. Biochem et. Biophys. Acta 9 65, 1952.

Fujita, A., Numata, I. Biochim. Z. 299 249, 1938.

G. Gartler, S.M. Arch. Biochem. & Biophys. 80, 400, 1959

Gelotte, B. J. Chromatog. 3, 330, 1960.

Gillette, R.W., Mansberger, A.R., Oppenheimer, J.H., Caldwell, B.F., Neff, E.G. Surgery, 43 747, 1958.

Giri, K.V., Sathyanarayana, Rao, G.J., Natatajan, S. Nature 182 184, 1958.

Goldblum, R.W., Piper, W.N., Olsen, C.J. J. Invest. Dermat. 22, 373, 1954.

Gordon, J., Jencks, W.P., Biochem. J. 2, 47, 1963.

Gornall, et al., J.B.C. 177, 759, 1949.

Green, H.N., Stoner, H.B., Whiteley, H.J., Eglin, D. Clin. Sci. 8, 65, 1949.

Grinert, R.R., Phillips, P.H., Arch. Biochem. 30, 217, 1951.

Guillemin, R., Clayton, G.W., Smith, J.D., Lipscomb, H.S. Endocrin 63, 349, 1958.

Guillino, P. et al., Arch. Biochem. & Biophys. 58, 255, 1950.

_____ Arch. Biochem. & Biophys. 64, 319, 1956.

H. Haden, R.L., J. Biol. Chem. 56, 469, 1923.

Hanzal, R.F., Proc. Soc. Exp. Biol. Med. 30, 846, 1933.

Hardy, J.D., Turner, M.D. Surgery 42, 194, 1957.

Harris, L.J., Proc. Royal Soc. Vol. 94B. 1923.

Hasner, E., Buus, J., Rygg, T., Therkelsen, J. Journ. Oslo City Hospital, 103, 11/12, 1961-62.

Hier, J., Trans. N.Y. Acad. Sci. 10, 280, 1948.

Holleman, R., De Bruyn, G. Brit. Exp. Research, 40, 3334, 1907.

Huggins, C., et al., Nature, 167, 592, 1951.

Hughes, W.L., Jr. Cold Spring Harbour Symposia Quant. Biol 14, 79, 1949.

Huisman, T.H.J., Benesch, R., et al., Sulphur in Proteins. Acad. Press. N.Y. p. 153, 1959.

Hume, D.M., Bell, C.C. Surg. Forum. 9, 6, 1959.

Humphrey, J.H., Jacques, R. J. Physiol. 124, 305, 1954.

Husfeldt, E. J. Oslo City Hosp. p. 99, 11, 1961.

I. Iber, F.L., et al., J. Lab. Clin. Med. 50, 417, 1957.

Ingbar, H., Kass, D. Soc. Exp. Biol. & Med. 77, 74, 1951

Ingram, V.M. Biochem. J. 59, 653, 1955.

J. Jarnum, S. Gastro. Ent. 41, 106, 1961.

Jocelyn, P.C. Biochem. J. 85, 480, 1962.

K. Karnin, H., Handler, P. Am. J. Physiol. 164, 654, 1951.

Katyal, J.M., Gorin, C. Arch. Biochem. & Biophys. 82, 319, 1959.

Katz, A.M., Maxwell, J.B. Circulation Research. 14, 345, 1964.

Kauzmann, W. Adv. Prot. Chem. 14, 1, 1959.

Kauzmann, W., Simpson, R.B. J. Am. Chem. Soc. 75, 5154, 1953.

Kauzmann, W., Frensdorff, H.K. et al., ibid. 75, 5167, 1953.

Killander, J., Flodin, P. Vox Sanquinis. I. No. 1. 113, 1962.

Kolthoff, I.M., Harris, W.E. Indus. & Eng. Chem. Anal. Ed. 18, 161, 1946.

Kohn, J. Biochem. J. 65, 9, 1957.

_____ Clin. Chim. Acta. 3, 450, 1958.

_____ Clin. Chim. Acta. 2, 297, 1957.

_____ Nature 181, 839, 1958.

L. Langemann, H. Helv. Physiol. Acta. 2, 367, 1964.

Lee, W.H., Krumharr, D., Fonkalsrud, E., Schjeide, O.A., Maloney, J.V. Surgery, 50, 29, 1961.

Lewis, G.P. (Ed.) 5 Hydroxytryptamine-Proc. a Symposium Pergamon Press April 1957.

Lewis, H.B. J. Biol. Chem. 68, 20, 1925.

Li, C.H., Gerschwind, I, Evans, H.M. J. Biol. Chem. 177, 91, 1949.

Liddle, G.W., Island, D., Meador, C.K. Recent Prog. in Hormone Research XVIII, 125, 1962. Acad. Press.

Lipscomb, H.S., Nelson, D.H. Endocrin. 71, 13, 1962.

Lovelock, J.E. Biochem. J. 60, 692, 1954.

_____ Nature Lond. 173 659, 1954.

M. McFarlane, A.S., Campbell, R.M., Cuthbertson, D.P., 1960. Unpublished data Biochem. response to injury, p. 202, Blackwell, Oxford.

Malloy, G., Evelyn, J., J. Biol. Chem. 119, 481, 1937.

Maltingly, D., Tyler, C. Proc. Royal Soc. Med. 58, 1010, 1965.

Marrian, D.H. J. Chem. Soc. 1515, 1949.

Mengel, C.E. Ann. of Inter. Med. 62, No. 3, 587, 1965

_____ et al., Amer. J. Med. 38, 396, 1965.

_____ Canc. Chemotherap. Reports, 51, No. 4, 239, 1967.

Millican, R.C. Biochem. Response to Injury, 269-290, 1960, Blackwell, Oxford. H.B. Stoner & C.J. Threfall Eds.

Mirsky, A.E., Anson, M.L. J. Gen. Physiol. 19, 427, 1935.

Mollison, D. Blood Transfusion in Clin. Med. (Blackwell) p. 568.

Moore, F.D., Ball, M.R. Metabolic Response to Surgery Thomas, Springfield, Illinois, 1952.

Moore, J., Stein, W.H. J. Biol. Chem. 150, 113, 1943.

_____ J. Biol. Chem. 192, 663, 1951.

Mouridsen, H.T. Clin. Sci. 33, 345, 1967.

Mudge, G.H. Amer. J. Med. 24, 785, 1958.

Munson, P.L., Toepel, W. Endocrin 63, 785, 1958.

N. Nardi, G.L. J. Clin. Invest. 33, 847, 1954.

Nelson, D.H., Hume, D.M. Endocrin 57, 184, 1955.

Neville, W.E. Current Problems in Surgery, July 1967.

Newberg, C., Lustig, H., Mandl, I. Arch. Biochem. 26, 77, 1950.

Newburgh, L.H., Marsh, P.L. Arch. Intern. Med. (Chicago) 36, 682, 1925.

Ney, R.L., et al. J. Clin. Invest. 42, 1669, 1963.

Norberg, B., et al. Acta. Chir. Scand. 120, 237, 1960.

Norlander, O., et al. Acta Anaesth. Scand. 2, 181, 1958-N.L.U.

O. Ouchterlony, O. Prog. Allergy, 5, 26, 1958.

Owens, W., Adams, J., McEhannon, A., Youngblood, S. J. App. Physiol. 14, 947, 1959.

P. Paline, V.G., et al. Aizneimittel-Forschung, p. 1034, Dec. 1963.

Pearse, A.G.E., Pepler, W.J. Nature, Lond. 179, 589, 1957.

Peterson, R.E., Kairer, A., Guerra, S.L. Anal. Chem. 29, 144, 1957.

Pincus, G. J. Clin. Endocrin. 3, 195, 1943.

Poulik, M.D., Smithies, O. Biochem. J. 68, 636, 1958.

Purves, H.D., Sirett, N.E. Endocrin. 77, 366, 1965.

Q. Quensel, W., Wacholder, K.Z. Physiol. Chem. 231, 65, 1935.

R. Resnik, R.A., Wolff, A.R. Arch. Biochem. Biophys. 64, 33, 1956.

Reynolds, R.C., Chenoweth, M.D., Ellman, G.L. Proc. Soc. Exp. Biol. Med. 99, 185, 1958.

Riggs, A., Wells, M. Federation Proceedings, 19, 78, 1960.

Rodnight, R. Biochem. J. 64, 621, 1956.

Ronzoni, E., Roberts, E., Frankel, S., Ramasarmo, G.B. Proc. Soc. Exp. Biol. Med. 82, 496, 1953.

Rubin, R. Personal Communication - New York City Hospital.

Russell, J.A., Wilhelmi, A.E. J.B.C. 137, 713, 1941.

Russell, J.A., Long, C.N.H., Engel, F.L. J. Exp. Med. 79, 1, 1944a.

Russell, J.A., Long, C.N.H., Wilhelmi, A.E. J. Exp. Med. 79, 23, 1944b.

_____ Am. J. Physiol., 147, 175, 1946.

S. Saenko, T.V., Ukrain Biokhim. Zhur. 24, 196, 1952.

Sandler, M., Snow, P.J.D. Lancet 1, 137, 1958.

Sayer, G. Hormonesin Blood, Vol. I, 2nd Edit., 169.

Sayers, M.A., Sayers, G., Woodbury, L.A. Endocrin. 42, 379, 1948.

Schachmann, H.K., Ultracentrifugation in Biochemistry, Acad. Press N.Y. 1959.

Schneckloth, R.E., Page, I.H., Del Greco, F., Corcoran, A.C. Circulation, in Press, 1957.

Schoken, K. Science, 116, 544, 1952.

Schroeder, E.F., Woodward, G.E. J. Biol. Chem. 129, 283, 1939.

Schwarzenbach, G. Hew. Chim. Acta, 29, 1338, 1946.

Scriver, C.R., Shafer, I.A., Efron, M.L. Nature, Lond. 192, 672, 1961.

Sheffner, A.L., Kirsner, J.B., Palmer, W.L. J. Biol. Chem. 175, 107, 1948.

Sheffner, A.L., Kirsner, J.B., Palmer, W.L. J. Biol. Chem. 176, 89, 1948.

_____ J. Clin. Invest. 28, 716, 1949.

Shore, B., Shore, V.G. Plasma 2, 621, 1954.

Shu'ayb, W.A., Noran, W.H., Zimmermann, B. Annals of Surgery, 162, No. 4, 690, 1965.

Silber, R.H., Busch, R.D., Oslapas, R. Clin. Chem. 4, 278, 1958.

Simko, J.P. Jr., Kauzmann, W. Biochem. I. 1005, 1962.

Simmons, D.J., Nichols, G. Amer. J. Physiol. 210, 411, 1966.

Simpson, M. J. Biol. Chem. 201, 143, 1953.

Simpson, R.B., Saroff, H.A. J. Amer. Chem. Soc. 80, 2129 1958.

Sjoerdsma, A., Weissbach, H., Udenfriend, S. Amer. J. Med. 20, 520, 1956.

Sjoerdsma, A., Waalkes, T.P., Weissbach, H. Science (a) In Press, 1957.

Smith, I. Chromatographic & Electrophoretic Techniques, Vol. I., 40, 1960, Locarte Comp.

Snow, P.J.D., et al. Lancet 11, 1004, 1955.

Spackman, D.H., et al. Anal. Chem. 30, 1190, 1958.

Starbury, et al. Metabolic Basis of Inherited Disease.

Stegmann, H. Z. Physiol. Chem. 311, 41, 1958.

Stein, W.H., Moore, S. J. Biol. Chem. 190, 103, 1951.

_____ J. Biol. Chem. 201, 45, 1953.

Stein, W.H., Bearn, A.G., Moore, S. J. Clin. Invest. 33, 410, 1954.

Stein, W.H., Moore, S. J.B.C. 211, 915, 1954.

Stephenson, S.E., Sawyers, J.L., Holcomb, G.L., Golian, F. Daniel, R.A., Scott, H.H.W. Surg. Forum. 42nd Clin. Congress 7, 257, 1957.

Sterling, K., et al. Metab. 4, 343, 1955.

Steven, F.S., Tristram, G.R. Biochem. J. 70, 2, 179, 1958.

Strickler, H.S., Grauer, R.C., Caughey, M.R. Arch. Biochem. & Biophys. 64, 88, 1956.

Sullivan, M.X. J. Biol. Chem. 50, XXXIX, 1922.

Sutton, W., Vandenberg, P. Hum. Biol. 25, 318, 1953.

T. Tallon, et al. J. Biol. Chem. 217, 703, 1955.

Thompson, A. Biochem. J. 60, 507, 1954.

Tristram, G.R., Smith, R.H. Adv. Prot. Chem. 18, 227, 1963.

Tsao, T.C., Bailey, K. Biochim et. Biophys. Acta. 11, 102, 1953.

U. Udenfriend, S., Weissbach, H., Clark, C. J. Biolchem. 215, 337, 1955.

Udenfriend, S., Titus, E., Weissbach, H. J. Biolchem. 216, 499, 1955.

V. Van Slyke, D.D., Hawkins, J.A. J. Biolchem. 79, 739, 1928.

Van Slyke, D.D., MacFadyen, D.A., Hamilton, P.B. J. Biolchem. 150, 251, 1943.

_____ Phillips, R.A., Hamilton, P.B., Archibald R.M., Dole, V.P., Emerson, K. Trans. Assoc. Am. Physicians. 58, 119, 1944.

Vernikos-Danellis, J., Anderson, E., Trigg, L. Endocrin. 79, 624, 1966.

W. Wacholder, K., Anders, K., Uhlonsbrook, K.Z. Physiol. Chem. 233, 181, 1935.

Walker, W.F. Proc. Royal Soc. Med. 58, No. 12, 1015, 1965.

Walker, W.F., Morgan, H.G., Breckenridge, I.M., Watt, A., Ogilvie, R.R., Douglas, D.M. Scottish Med. J. 8, 141, 1963.

Walton, K.W., Scott, P.J. J. Clin. Path. 17, 627, 1964.

Weissman, N., Schoenbach, E.B., Armstead, E.B. J. Biol. Chem. 187, 153, 1950.

Werner, M., Cohnen, G. Clin. Science, 36, 173, 1969.

Westall, R.G. Biochem. J. 52, 638, 1952.

_____ Biochem. J. 60, 247, 1955.

Wolfson, W.Q., Cohn, C., Calvary, E., Ichiba, F. Amer. J. Chem. Path. 19, 723, 1948.

Wong, S.Y. J. Biol. Chem. 77, 409, 1928.

Woodward, G.E. J. Biol. Chem. 97, 465, 1932.

Wright, E.S., Sarkozy, E., Harper, E.R., Dobell, A.R.C., Murphy, D.R. J. Thor. & Cardiovas. Surg. 44, No. 4, 550, 1962.

Wronski, M. Biochem. J. 104, 978, 1967.

Y. Yalow, R.S., Berson, S.A. Brit. J. Hosp. Med. 2, No. 7, 1300, 1969.

Yalow, R.S., Glick, S.M., Roth, J., Berson, S.A. J. Clin. Endocrin. Metab. 24, 1219-1964.